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13. ABSTRACT (Maximum 200 Words)

Previous research from this laboratory indicated that 1. the expression of Tropomyosin-1 (TM1), a microfilament-associated protein, is abolished from many human breast carcinoma cells, and; 2. that TM1 is suppressor of the malignant transformation. These data led to the hypothesis that TM1 plays an important role in mammary carcinogenesis. Therefore, we investigated whether TM1 could serve as a biomarker of breast cancer, and TM1 could function as a suppressor of the malignant growth of breast cancer cells. Our results show that TM1 is downregulated in breast tumors (Objective 1). We demonstrated that TM1 is a suppressor of the malignant growth phenotype of MDA MB 231 cells, indicating that TM1 is a general suppressor of the transformed growth (Objective 2). To assess the structure-function relationship of tumor suppression by TM1, we constructed chimeric and variant TM1 proteins. By employing a variant TM1 that contains an aminoterminal extension, we show that the aminoterminal integrity is important for TM1-mediated tumor suppression (Objective 4).

14. SUBJECT TERMS
Tropomyosin, biomarker, tumor suppressor, diagnosis/prognosis

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Introduction

The presence of disorganized or poorly structured microfilaments is a prominent feature of many transformed cells. Disorganized cytoskeleton is thought to contribute to the malignant growth of cells. Microfilaments regulate cell division, cell motility and intracellular transport. However, the role of microfilament-associated proteins in neoplastic transformation remains largely unclear. Downregulation of microfilament-associated proteins, such as tropomyosins (TMs) is hypothesized to result in the formation of functionally aberrant microfilaments, thus contributing to the manifestation of malignant cells. TMs are a family of cytoskeletal proteins that bind to and stabilize actin. This research proposal is based on the preliminary results which have identified that: 1) Tropomyosin-1 (TM1) is a suppressor of the transformed phenotype, and; 2) TM1 is consistently abolished in a large number of breast carcinoma cells that are tested. The main objectives of the proposed research are to assess the expression of TM1 in the tissue specimens of breast cancer patients and to investigate whether TM1 functions as a suppressor of the malignant growth of breast cancer. We have accomplished the proposed goals of the project.

Final Summary

Technical Objective 1: Analysis of TM1 expression in human malignant breast tumors and benign lesions, and normal breast tissues:

Normal mammary epithelial cells elaborate multiple isoforms of TMs [1, 2]. The spontaneously transformed breast cancer cells were shown to exhibit severe deficiency in TM expression, with the expression of several TMs is either downregulated or completely abolished. For example, TM38 protein is expressed in MDA MB231 cells, but found to be absent in MCF-7 cells. Expression of TM1, however, was consistently lacking in all of the breast carcinoma cell lines tested, indicating that TM1 suppression could be a common event during mammary carcinogenesis. Based on these data, we hypothesized that loss of TM1 is a critical biochemical change in the malignant transformation of breast epithelial cells and that TM1 could be used as a novel biomarker of breast cancer.

Since multiple TMs are present in epithelial cells, and the presence of stromal components which abundantly express TM1, we chose to analyze the tissues by immunohistochemistry. The smooth muscle cells of the blood vessels also express TM1. Currently available antisera recognize multiple isoforms of TMs and, thus, do not permit accurate analysis of TM1 expression. We have proposed to employ TM-specific antibodies to assess TM1 expression in breast tissues. We generated several TM1-specific antibodies in this lab. TM1-specific reactivity of these antibodies has been described in two recent publications included in the appendix.

In addition, we have developed a sensitive in situ hybridization method to determine TM1 expression in breast tissues. For this purpose, we collected normal and malignant breast tissue specimens from the patients undergoing surgery, as part of their clinical care. Tissue specimens were collected under IRB approval. We analyzed 24 normal and 25 invasive breast tumors for TM1 expression. While all of the normal tissues expressed abundant quantities of TM1, none of the breast tumors expressed significant levels of TM1. Quantitation of TM1 expression revealed profound differences between normal ducts and the invasive tumors. TM1 mRNA expression was quantitated by counting the intensity and number of the silver grains. Relative quantity of TM1 in normal tissue was at 29.7 units, while the malignant breast tissue expressed TM1 at 3.5 units. The signal obtained with the tumors was comparable to that obtained with the 'sense' control probes. These data are in agreement with those obtained with the cultured cells, and demonstrate that the expression of TM1 is profoundly suppressed in breast tumors [3].

In a second line of study to further determine changes in TM1 expression in breast tumors, we have employed immunofluorescence to determine changes in TM1 expression in breast tissues. We have employed the novel TM1 specific antibodies [2] to evaluate TM1 expression in breast tissues. Consistent with the results obtained with in situ hybridization data, TM1 protein was undetectable in 25 primary tumors, while all the normal tissues expressed TM1 [3].

We next screened several commonly employed lung and colon carcinoma cell lines for TM1 expression to determine whether loss of TM1 expression is specific for

breast cancer cell lines. These results indicated while some lung and colon carcinoma cells lack TM1, some contain detectable TM1, suggesting that a consistent loss of TM1 expression may be a specific feature of breast carcinoma cells.

Technical Objective 2: Effects of expression of TM1 in human breast carcinoma cell lines and in normal mammary epithelial cell lines.
Our previous studies with experimentally transformed murine fibroblasts have demonstrated that TM1 is a suppressor of the malignant transformation, and that TM1 is a class II tumor suppressor [1, 4]. To examine the role of TM1 in mammary carcinogenesis, and to determine whether TM1 can suppress the malignant growth of a spontaneously transformed human breast carcinoma cells, the following experiments were carried out. These data are communicated for publication, and the manuscript is attached in the appendix. Therefore, the results are briefly presented below.

MCF-7 cells, which lack TM1 protein, were transduced with a recombinant retrovirus expressing TM1. Individual cell lines expressing TM1 were isolated and tested for the effects of TM1 expression on the morphology and growth properties [2]. Restoration of TM1 expression resulted in the formation of tighter colonies with a more branched, tubular appearance. The presence of TM1 containing microfilaments is readily detected. TM1 expression significantly decreased the growth rates, compared to parental MCF-7 cells. A more profound effect was observed on the anchorage-independent growth property, which is a hall mark of the neoplastic phenotype. TM1 expression completely abolished the anchorage independent growth of MCF-7 cells, indicating that TM1 suppressed the malignant growth properties. It should be noted that the revertant cells remain sensitive to the growth control by estrogen. In a different approach, we have employed adenoviral vectors to transducer MCF-7 cells, and demonstrated that TM1 expression abolishes anchorage-independent growth.

With these experiments, a majority of the goals of this Objective are accomplished, and we completed the Objective 2. Additional experiments in progress are directed at elucidating the mechanism of tumor suppression by TM1.

Additionally, we demonstrate that TM1 suppresses the malignant growth properties of MDA MB 231 cells [2]. Thus, TM1 suppresses the malignant growth properties of breast cancer cells, regardless of their p53 and estrogen receptor status. These findings support the hypothesis that TM1 is a general suppressor of neoplastic growth.

A new line of work to investigate the mechanism of tumor suppression by TM1 is initiated. Since TM1 suppresses anchorage independent growth, we investigated whether TM1 induces anoikis (detachment induced apoptosis) in breast cancer cells. Initial studies indicate that TM1 sensitizes breast cancer cells to anoikis [3]. Further work on the mechanism of TM1-induced anoikis is in progress.

3. Induction of transformed phenotype by repression of TM1 of TM1 expression: In order to test whether the loss of TM1 expression could lead to the expression of malignant transformation of mammary epithelium, antisense suppression of TM1 is proposed. TM1 was subcloned in antisense direction in the retroviral vector pBNC and antisense packaging cells of PA317 are generated. The MCF10A cells have been transduced with

the recombinant pBNC retrovirus. Transduced cells were selected for resistance to G418 and single cell clones were selected. These cell lines were tested for growth under anchorage independent conditions. MCF10A cells expressing antisense TM1 were unable to grow under anchorage independent growth conditions, indicating that antisense expression was not adequate to suppress TM1 expression. These data indicate that either the antisense RNA is not effective in suppressing TM1 or, the antisense suppression of TM1 alone does not transform the breast cancer cells. We are investigating these possibilities. The work has progressed as per the Statement of Work.

At this point we are considering more effective and novel strategies for antisense suppression. For example, we are considering to employ RNAi approaches to block TM1 expression.

4. Structure-function relationship of TM1-mediated tumor suppression: Studies completed in Techinical Objective 2 have shown that TM1 is a suppressor of malignant growth. We next, tested the isoform-specificity of tumor suppression by TM1. For this purpose, we have created a cell line derived from MCF-7 cells that expresses TM2-a isoform that shares significant homology with TM1. We have subcloned TM2 cDNA into a plasmid vector that co-expresses green fluorescent protein via IRES sequences to mark the transfected cells. MCF-7 cells were transfected with TM2 plasmid and selected with the G418. Stable cells expressing TM2 were tested for their growth in agar. TM2 cells grew in anchorage independent conditions, indicating that TM2, unlike TM1, is not a tumor suppressor.

To test whether the amino and carboxy terminal portions of TM1 are responsible for tumor suppression, chimeras of TM1 (a tumor suppressor) and TM2 (not a tumor suppressor).We have completed the site directed mutagenesis to introduce a silent mutation to create a HindIII restriction site. This was accomplished by PCR and the resultant variants of TM1 and TM2, designated as 'TM1-TM2 and 'TM2-TM1' respectively. We have first tested the anti-oncogenic effects of the chimeras in a model cell line. We have utilized ras-transformed NIH3T3 cells. The results show that the amino terminus is critical for TM1-induced cytoskeletal reorganization and suppression of malignant growth.

At this point, in parallel, we have chosen to test the use of epitope-tagged TMs in a different cell system. Several investigators have used epitope-tagged constructs to monitor the transfected genes. Epitope tagging of TM1 is expected to greatly facilitate the analysis of transfected TMs. To confirm that epitope tagging does not impair the tumor suppressive function of TM1, we chose to test the constructs in DT cell (ras-transformed NIH3T3 cells) system. Our previous published studies showed that DT cells are suitable models to test the ability of TM1 to reorganize the cytoskeleton and tumor suppression. A HA epitope was engineered to the amino terminus of TM1. The ATG initiation codon of TM1 was replaced with an oligonucleotide sequence encoding HA epitope. The recombinant TM1 was subcloned into a eukaryotic expression vector, and transfected into DT cells.

The epitope tagged TM1 profoundly differed from the wild type protein in terms of its ability to function as a tumor suppressor. Transfection of epitope tagged TM1 did

not induce microfilaments, alter the growth rates or inhibit the growth in agar. These data show that modification of N-terminal portion of TM1 completely abolishes the tumor suppression. These results further support the importance of the amino terminus of TM1 in cytoskeletal reorganization and suppression of malignant growth.

Key Research accomplishments

- TM1 is downregulated in breast tumors
- Consistent downregulation of TM1 is a feature of breast cancer cells. Some lung and colon cancer cells, and melanoma cells express TM1.
- TM1 is a suppressor of MCF-7 and MDA MB 231 cells. This finding supports that TM1 is a general suppressor of cellular transformation.
- TM1 is downregulated by gene methylation and histone deacetylation.
- N-terminal modification of TM1 abolishes the tumor suppression
- TM2 is not a tumor suppressor.
- TM1 induces anoikis in breast cancer cells.

Report date: October 14, 03.

Reportable Outcomes

Manuscripts and Abstracts

1. Manuscripts:

1.1. S. Bharadwaj and G. L. Prasad (2002) Tropomyosin-1 is downregulated by Promoter Methylation in Cancer Cells. Cancer Letters 183: 205-213.

1.2. Suppression of transformed phenotype of breast cancer by tropomyosin-1 (2002). Kalyankar Mahadev, Gira Raval, Mark Willingham, Ethan M. Lange, Barbara Vonderhaar, David Salomon, and G. L. Prasad. Experimental Cell Research 279: 40-51. This paper has been selected by the Editor for presentation in the highlight section of the journal.

1.3. Loss of expression of Tropomyosin-1, a Novel Class II Tumor Suppressor that induces anoikis, in Primary Breast Tumors (2003). Gira N. Raval1, Shantaram Bharadwaj1, Edward A. Levine, Mark C. Willingham, Randolph L. Geary, Tim Kute and G. L. Prasad. Oncogene 6194-6203. 1Co-first authors

1.4. Shantaram Bharadwaj, Sarah-Hitchcock-DeGregori Andrew Thorburn and G. L. Prasad. Amino Terminus is Essential for Tropomyosin-1 Mediated Reorganization of Microfilaments and Suppression of Malignant Growth. (manuscript submitted).

2. Abstracts

2.1. Tropomyosin-1 is a tumor suppressor and a marker of breast cancer Mahadev Kalyankar, James G. Caya, Barbara K. Vonderhaar¶, and G. L. Prasad. (1999) 39th Annual meeting of the American Society for Cell Biology, Washington DC #L50.

2.2. Tropomyosin-1 is a suppressor of the malignant phenotype of breast cancer G. L. Prasad and Mahadev Kalyankar (2000) Era of Hope meeting, Atlanta. #B77.

2.3. Cytoskeletal Proteins as Markers and Suppressors of the Neoplastic transformation: Studies on Tropomyosin-1. G. L. Prasad, S. Bharadwaj and G. Raval (2000). 20th annual convention of Indian Association for Cancer Research. Ahemdabad, India.

2.4. Tropomyosin-1 is a suppressor of the malignant phenotype of breast cancer cells. (2001) G. L. Prasad S. Bharadwaj, G. N. Raval, and B. K. Vonderhaar. Gordon conference on Mammary Gland Biology.

2.5. Tropomyosin-1, a novel tumor suppressor and a biomarker.Gira Raval, Shantaram Bharadwaj, Edward Levine1, Mark Willingham2, Randolph Geary1 and G. L. Prasad. Era of Hope, Army Breast Cancer Meeting. Orlando, FL, September 2002.

2.6. Molecular Basis of Suppression of the Malignant Growth Phenotype by Tropomyosin-1. S. Bharadwaj, G. L. Prasad. ASCB meeting: San Francisco, Dec: 14-18, 2002.

2.7. Cytoskeletal Proteins as Regulators of Breast Cancer: Anoikis and Tumor Suppression by Tropomyosin-1, a Microfilament Stabilizing Protein. Shantaram Bharadwaj, Edward A Levine and G. L. Prasad. AACR Special Meeting

Advances in Breast Cancer Research Genetics, Biology and Clinical Implications, October 8-12, 2003, Huntington Beach, CA.

Cell lines and reagents:

1. TM1 specific antibody
2. MCF-7 and MDA MB 231 cells expressing TM1
3. MCF-7 cells expressing TM2
4. Adenoviral vectors that express TM1.
5. Chimeric Tropomyosins, and a variant TM1 molecule

Grants:

Several grant applications are now pending

Promotions:

The PI is now promoted to the rank of Associate Professor based on his funding from DOD

The U.S. Army Medical Research and Materiel Command under DAMD-99-1-9395 supported this work.

Conclusions

In summary, the major accomplishments of our work for are two fold: First, we demonstrated, for the first time, that TM1 is downregulated in breast tumors. While TM1 has been known to bind and stabilize actin microfilaments for a long time, its role in modulating tumor growth is now becoming evident. Second, we have demonstrated that TM1 is a suppressor of the malignant growth of breast cancer. The discovery that TM1 expression is significantly downregulated in invasive breast tumors suggests TM1 could be used as a novel biomarker.

There is a significant increase in the detection of smaller breast masses due to sensitive mammographic techniques, and it is necessary to determine whether they are benign and have the potential to become malignant. A suitable molecular marker, such as TM1, which is altered only in malignant tissues may be a useful surrogate marker in this regard. Continued expression of TM1 at significant levels would indicate that the tissue is benign, or a decrease in TM1 expression would suggest a need for further evaluation.

TM1 may also serve as a marker to determine the endpoint or efficacy of novel therapies involving inhibition of DNA methyl transferases and histone deacetylases [5]. Since TM1 gene is silenced by gene methylation and histone deacetylation, these therapies would upregulate TM1 expression and other key genes that are essential for normal growth [5]. Upregulated TM1 may serve as a marker for the effectiveness of the drugs, and reactivated TM1 itself may contribute to the suppression of malignant growth, as shown in the culture experiments [2].

The finding that TM1 induces anoikis [3] may explain why TM1 is downregulated in primary breast tumors. The resistance to anoikis is considered to be a essential for metastatic growth [6]. Furthermore, we also find that TM1 inhibits binding of breast cancer cells to collagen I matrices specifically (work in progress). Binding to collagen is considered to be a necessary for efficient invasion of bone [7]. Therefore, investigations into how TM1 decreases binding to collagen may lead to better strategies to treat breast cancer. Work along those lines is in progress.

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List of Personnel Supported by this Award

1. G. L. Prasad, PI
2. Shantaram Bharadwaj, Research Associate

APPENDIX COVER SHEET

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Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors

Gira N Raval[1,3], Shantaram Bharadwaj[1,3], Edward A Levine[1], Mark C Willingham[2], Randolph L Geary[1], Tim Kute[2] and GL Prasad*[,1]

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Suppression of tropomyosins (TMs), a family of actin-binding, microfilament-associated proteins, is a prominent feature of many transformed cells. Yet it is unclear whether downregulation of TMs occur in human tumors. We have investigated the expression of tropomyosin-1 (TM1) in human breast carcinoma tissues by *in situ* hybridization and immunofluorescence. TM1 mRNA and protein are readily detectable in normal mammary tissue. In contrast, TM1 expression is abolished in the primary human breast tumors. Expression of other TM isoforms, however, is variable among the tumors. The consistent and profound downregulation of TM1 suggests that TM1 may be a novel and useful biomarker of mammary neoplasms. These data also support the hypothesis that suppression of TM1 expression during the malignant conversion of mammary epithelium as a contributing factor of breast cancer. In support of this hypothesis, we show that the ability to suppress malignant growth properties of breast cancer cells is specific to TM1 isoform. Investigations into the mechanisms of TM1-induced tumor suppression reveal that TM1 induces anoikis (detachment induced apoptosis) in breast cancer cells. Downregulation of TM1 in breast tumors may destabilize microfilament architecture and confer resistance to anoikis, which facilitates survival of neoplastic cells outside the normal microenvironment and promote malignant growth.
Oncogene (2003) **22,** 6194–6203. doi:10.1038/sj.onc.1206719

Keywords: tropomyosin; breast cancer; tumor suppressor; anoikis; biomarker

Introduction

During neoplastic transformation, cells undergo numerous biochemical changes, some of which confer a selective advantage over their normal counterparts to facilitate malignant growth. These changes include mutational inactivation of tumor suppressor proteins, activation of oncogenes and other epigenetic events (Fearon and Vogelstein, 1990; Beckmann *et al.*, 1997). Elucidation of these causal molecular events is necessary to develop better tumor markers and identify effective targets for cancer treatment. A number of research groups have demonstrated that suppression of high-M_r tropomyosin (TM) isoforms (tropomyosin-1 and tropomyosin-2) occurs in malignant cells, suggesting a role for these proteins in neoplastic transformation (Hendricks and Weintraub, 1981; Matsumura *et al.*, 1983; Cooper *et al.*, 1985; Bhattacharya *et al.*, 1990).

TMs are a family of actin-binding proteins that stabilize microfilaments and are expressed with a high degree of tissue specificity (Lin *et al.*, 1997; Pawlak and Helfman, 2001). TMs may be broadly categorized into high- and low-M_r species depending on their size. In nonmuscle cells, such as fibroblasts and epithelial cells, multiple high-M_r TMs are expressed, which are referred to as isoforms TM1, TM2 and TM3; additionally, epithelial cells also express another protein designated as TM38. Similarly, smooth muscle cells express two TM isoforms, TM1 and TM2. Previous work from this laboratory has demonstrated that multiple TM isoforms are downregulated in human breast carcinoma cells (Bhattacharya *et al.*, 1990). More significantly, expression of TM1 was consistently abolished in most commonly studied breast cancer cell lines, indicating that loss of TM1 could be an important event in mammary carcinogenesis. This finding is in line with the general observations that most neoplastic cells exhibit altered cellular morphology and contain disorganized microfilaments, and that suppression of TM1 is a common biochemical event in cells transformed by diverse oncogenic modalities (Cooper *et al.*, 1985). We hypothesized that loss of TM1 results in the formation of disorganized microfilaments, which in turn, facilitate neoplastic conversion. Consistent with this hypothesis, TM1 reorganizes actin filaments and functions as a supp-ressor of malignant transformation (Prasad *et al.*, 1993, 1999; Braverman *et al.*, 1996; Mahadev *et al.*, 2002).

Since many breast carcinoma cell lines lack TM1, we considered whether TM1 could serve as a potential tumor marker. Two previous studies, utilizing biochemical methods, have suggested that TM1 expression may be enhanced in breast tumors (Franzen *et al.*,

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1996, 1997). However, we believe that accurate assessment of TM1 expression in tissues by biochemical methods is difficult for several reasons. For example, (1) multiple TM proteins share extensive sequence homology; (2) TM1 is expressed in multiple cell types present in tissues. In addition to epithelial and stromal cells, smooth muscle cells of tumor vasculature express abundant amounts of TM1 and; (3) the lack of isoform-specific antibodies has rendered the accurate assessment of TM1 protein by immunohistochemistry in human tissues difficult. In this communication, we have investigated whether TM1 expression is altered in human breast carcinomas, and the isoform specific breast cancer suppression by TMs. We also report that inhibition of malignant growth of breast cancer cells by TM1 involves induction of anoikis.

Results

Since TM1 expression is consistently lost in breast cancer cells, and TM1 suppresses the malignant growth of breast cancer cells (Mahadev *et al*., 2002), we have chosen to investigate changes in TM1 expression in breast cancer. We utilized *in situ* hybridization and immunofluorescence methods to validate the RNA probes and TM1-specific antibody (data not shown) using MCF10A and MCF-7 cells, which contain known TM profiles (Bhattacharya *et al*., 1990). TM1 expression was detected in MCF-10A cells; MCF-7 cells, on the other hand, lacked TM1 mRNA and protein, in agreement with the Northern blotting data and two-dimensional gel analyses.

TM1 mRNA expression is downregulated in breast cancer

To determine whether TM1 expression is altered in breast tumors, *in situ* hybridization was performed. Adjacent normal tissues from the breast cancer patients, as judged by gross examination and histology, were also used. TM1 expression was readily detectable by the intense labeling of silver grains in the normal ducts (Figure 1). Adjacent sections hybridized to the control sense probe lacked specific signal and pattern, and was diffuse and weak.

Analysis of the adjacent invasive tumor tissue revealed profound differences in TM1 expression compared to the normal tissue. The invasive breast tumor tissue lacked any detectable TM1 mRNA, and the signal obtained with the antisense probe and the control sense probe was essentially identical. The signal obtained with the antisense probe in tumor tissue was comparable to that obtained with the background signal. Analysis of five different normal ducts and malignant cells revealed significant differences between the tissues. The mean intensity, indicative of the abundance of TM1 mRNA in normal ducts, was 29.7 ± 8.1 (mean $\pm$ s.d.) after subtracting the background signal obtained with the sense probe. In contrast, TM1 mRNA levels in the tumor tissue corresponded to a mean value of 3.8 ± 2.8 over the background. Thus, TM1 expression is profoundly suppressed in breast tumors.

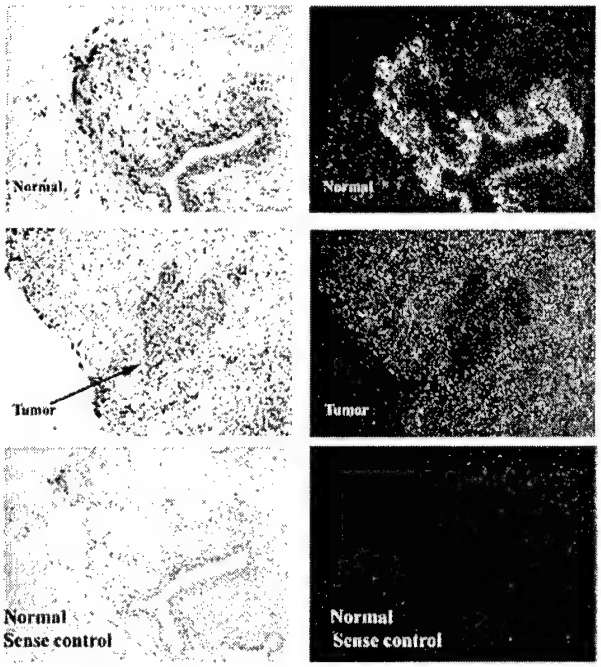

Figure 1 TM1 mRNA expression in normal and malignant breast tissue: normal and malignant breast tissues were hybridized with antisense (top and middle panels) and sense TM1 probes (bottom panel), and developed as described in Materials and methods. Samples were photographed in bright field to view the tissue architecture, and in dark field to view the silver grains indicative of TM1 expression. H&E staining of the tissue, sense probe hybridization of tumor tissue are not depicted. Samples were photographed at $\times 25$ magnification

Immunohistochemical analysis of TM1 expression in invasive breast cancer

To further evaluate changes in TM1 expression, we have performed immunohistochemistry on the same set of tissues used for *in situ* studies.

Immunofluorescence analysis of normal breast epithelium with a TM1-specific antibody (Mahadev *et al*., 2002) revealed specific staining of both myoepithelial and luminal epithelial layers of the ducts for TM1 (Figure 2a). Omission of the primary antibody in the reaction (background) did not produce any detectable staining. Autofluorescence in the FITC channel revealed background signal that did not interfere with the TM1 signal.

Malignant breast epithelium, on the other hand, lacked any detectable TM1, and the signal intensity in the tumor area was equal to that obtained with omission of the primary antibody (Figure 2b). The blood vessels present in the tumor, however, stained intensely with TM1 antibody, indicating that the lack of staining in tumor tissue was not due to the inability of the antibody

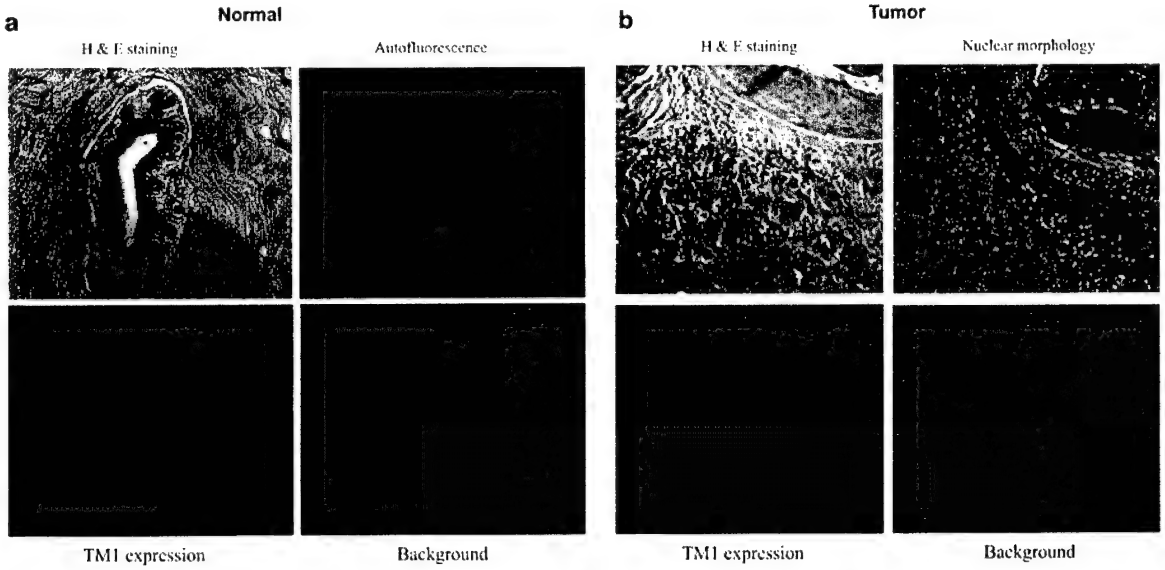

Figure 2 Expression of TM1 protein in normal and malignant breast tissues: (**a**) TM1 is expressed in normal ducts. Tissue sections were stained with TM1-specific antibody, and TM1 staining was viewed with a rhodamine cube, or autofluorescence was recorded through FITC cube. A parallel section was processed by omitting the primary antibody for negative control (background). H&E staining of the tissue sample is also shown. (**b**) Tumor tissue lacks expression of TM1: breast tumor stained with TM1-specific antibody (TM1 expression), negative control (background) and nuclear morphology along with H&E staining are shown. While the tumor area lacks TM1 staining, a blood vessel in the tumor reacts strongly, serving as a positive control for TM1 staining. ×25 magnification

to detect TM1. TM1 expression was undetectable in any of the 25 tumors tested, independent of any other parameters such as the stage, nodal status, hormone receptor status, proliferative index or HER2/neu status.

Quantitation of TM1 expression in tumor tissues revealed a significant decrease compared with the normal tissue. We measured relative luminosity in 10 normal and nine tumor tissue images, along with 14 background images taken from different tissues. Based on results from a mixed model, the adjusted mean levels of luminosity in normal tissues are 47.5 ± 1.1, for tumor tissues 16.2 ± 1.1, and the background signal is 15.3 ± 1.1 (mean $\pm$ s.e.). TM1 expression levels in normal tissues over the background are highly significant ($P < 0.0001$). The difference in TM1 protein levels between normal and tumor tissues is highly significant ($P < 0.0001$), indicating a profound downregulation of TM1 in breast tumors. However, the difference between tumor and the background signal was not significant ($P = 0.63$). These results demonstrate that while normal mammary tissues express abundant quantities of TM1, the malignant breast tumors essentially lack TM1.

To investigate, whether in addition to TM1, other TMs are also downregulated in breast tumors, tissue sections were screened with a pan-TM antibody which reacts preferentially with the high-M_r TMs, including TM1 (Bhattacharya *et al*., 1990; Mahadev *et al*., 2002). The normal ducts were intensely stained with the pan-TM antibody, indicating the expression of TM1 and other TMs in both the basal and luminal epithelial cells (Figure 3a). In breast tumor specimens, however, the staining pattern was variable. In most tumors (19/25, 76%) no signal was detected, indicating lack of or low level of expression of TMs (Figure 3b; Table 1). Blood vessels present in tumor tissue intensely stained, demonstrating the reactivity of TM antibody.

In a significant number (6/25 samples; 24% of breast tumors; Table 1) of tumor tissues, the pan-TM antibody reactivity was readily evident by the intense staining of tumor tissues, indicating the expression of other TM proteins (Figure 3c). The same set of samples stained negative for TM1 expression in parallel experiments, indicating that TM1 was consistently abolished in breast tumors, and expression of other TMs was variable. The widespread suppression of TM1 in breast carcinomas and the ability of TM1 to inhibit malignant growth of breast cancer cells strengthens the hypothesis that TM1 is a tumor suppressor (Prasad *et al*., 1993, #5; Prasad *et al*., 1999, #6; Mahadev *et al*., 2002, #41). Taken together, our findings suggest that the loss of TM1 expression is a crucial event that directly contributes to mammary carcinogenesis.

Isoform-specific suppression of malignant growth of breast cancer cells by TM1

Since both TM1 and TM2 share extensive sequence homology (Figure 4a), and contrary to our findings (Braverman *et al*., 1996), TM2 is suggested to function as a ras-suppressor (Janssen and Mier, 1997), we have tested the effect of restoration of TM2 in breast cancer cells. Stable expression of TM2 failed to inhibit the anchorage-independent growth of MCF-7 cells (Figure 4b). Both parental MCF-7 cells and those

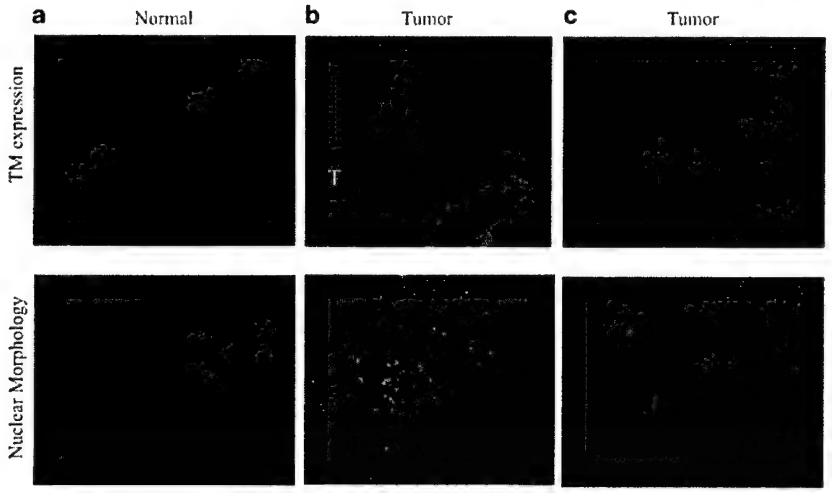

Figure 3 Expression of other TM isoforms is variable in breast tumors. Tissues were stained with a pan-TM antibody that reacts with multiple TM proteins (top panels, labeled as TM expression). The samples were also reacted with DAPI to visualize nuclear morphology and tissue architecture (bottom panels, marked as Nuclear morphology) following the antibody incubations. **(a)** The pan-TM antibody detects TMs isoforms in mammary epithelium. **(b)** A tumor sample with no detectable TM staining. However, TMs expressed in a blood vessel are readily detected. Area of the tumor is identified with 'T' in the top panel. **(c)** A tumor sample with reactivity to pan-TM antibody

Table 1 TM expression in breast tumors

Tissues	*Number of tissues expressing*	
	TM1	*Other TMs*
Normal breast epithelium	All	All
Malignant breast tissue	0/25	6/25

expressing TM2 formed colonies in agar, indicating that TM2 is not a suppressor of transformation in breast cancer cells.

Adenoviral transduction of TM1, on the other hand, suppresses anchorage-independent growth of MCF-7 cells (Figure 4c). We have transduced MCF-7 cells using the AdEasy system, which allows for coexpression of green fluorescent protein (GFP) along with wild-type TM1 (AdTM1). TM1 expressing MCF-7 cells, visualized by GFP expression, failed to grow under anchorage-independent conditions, while those not infected with AdTM1 virus, or those transduced with the control virus expressing only GFP (AdGFP), grew aggressively. Since MCF-7 cells grow as clumps, during the initial culture period, GFP expressing clumps were evident, which diminished with time. At the end of 20 days of culture, essentially all of the TM1 expressing cells died, as observed by lack of GFP expressing cells. MCF-7 cells transduced with AdGFP virus yielded 4870 colonies of cells positive for GFP in this representative experiment. In contrast, transduction of MCF-7 cells with TM1 resulted in a 50% decrease in total number of colonies compared to those infected with the control virus, reflecting the 50% efficiency of the adenoviral transduction in this experiment. These data further support the isoform specificity of TM1-mediated suppression of the malignant growth properties of breast cancer cells (Braverman *et al*., 1996).

TM1 induces anoikis in breast cancer cells

In efforts to better understand TM1-mediated tumor suppression, we have investigated whether TM1 modulates cell cycle. As depicted in Figure 5a, asynchronous populations of MCF-7/TM1 cells contained significantly lower fraction of cells in S phase compared to MCF-7 cells. The number of cells in G_2–M phase was slightly higher in MCF-7/TM1 cells than in the parental cells. Furthermore, 5-bromo-2′-deoxyuridine (BrdU) incorporation studies (Figure 5b) also revealed that TM1 expression decreases the S phase in MCF-7 cells. The S-phase fraction values for MCF-7 cells (34.4%) were significantly higher than with MCF-7/TM1 (20.7%), indicating a lower rate of DNA synthesis. Interestingly, a significant number of MCF-7/TM1 cells, although were in S phase (based on DNA content), remained 'inactive' as judged by BrdU incorporation (Figure 5b). Thus, the slower monolayer growth of MCF-7/TM1 cells observed in previous studies (Mahadev *et al*., 2002) is due to a decrease in S-phase fraction, and does not fully explain tumor suppression by TM1.

One of the hallmarks of malignant growth is to acquire resistance to undergo apoptosis when deprived of normal cell–matrix interactions, also known as anoikis (Frisch and Screaton, 2001; Stupack and Cheresh, 2002). TM1 restoration in many transformed cells profoundly suppresses anchorage-independent growth, indicating that TM1 resensitizes malignant cells to adhesion-dependent survival. Therefore, we have further investigated whether TM1-mediated tumor suppression involves anoikis.

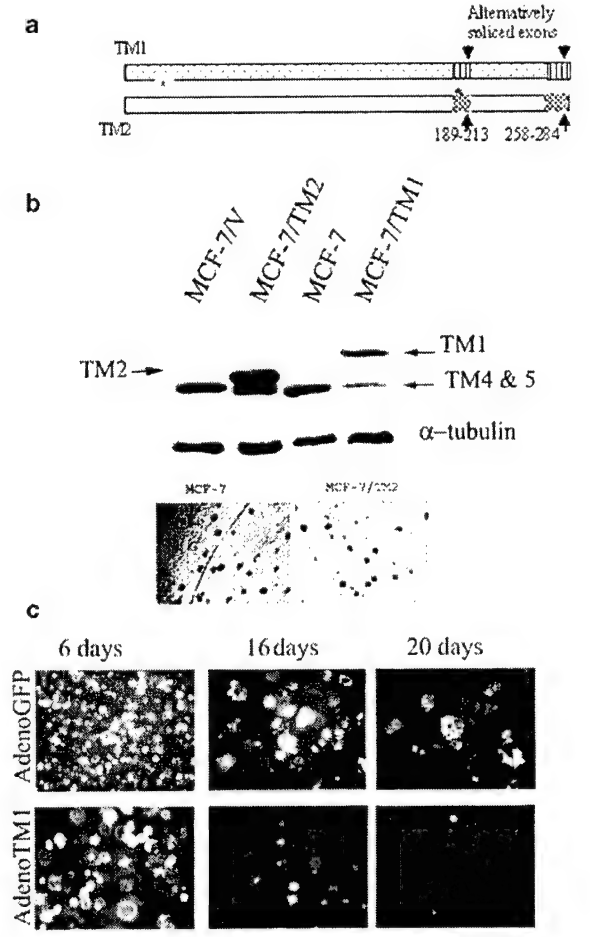

Figure 4 Isoform specificity of tumor suppression by TM1. (**a**) Comparison of TM1 and TM2 proteins. TM1 and TM2 share extensive sequence homology except in the sequence coded by two alternatively spliced exons (amino acids 189–213 and 258–284). The asterisks identify unique cysteine residues in TM1 (cys 35) and TM2 (cys 190). (**b**) TM2 is not a tumor suppressor of breast cancer cells: MCF-7 cells were transfected to restore the expression of TM2, and stably selected cells were tested for TM2 expression using a commercial antibody (TM311, Sigma). Unmodified MCF-7 cells, empty vector transduced cells (MCF-7/V), TM2 and TM1 expressing MCF-7 cells were used in the immunoblotting experiment (top). Parental and TM2 expressing MCF-7 cells were cultured under anchorage-independent conditions (bottom). (**c**) Adenoviral transduction of TM1 suppresses anchorage-independent growth of MCF-7 cells: control and TM1 expressing adenoviruses were used to infect MCF-7 cells at 50% efficiency for 24 h, and then 2×10^4 cells were plated on soft agar. Colony formation was monitored and the GFP-expression was recorded. TM1 expressing cells did not grow, as evidenced by lack of GFP signal, while the control vector transduction yielded a large number of GFP-positive colonies. Images were captured at $\times 10$ magnification using FITC cube. The bright field images are not shown. Results of a representative experiment are shown

MCF-7/TM1 and MDA MB 231/TM1 were cultured in suspension and tested for cell survival and apoptosis. Unmodified MCF-7 cells, and those transduced with TM1 were cultured on polyHEMA-coated dishes to prevent adhesion to substratum, and examined nuclear morphology to determine whether TM1 induces anoikis. MCF-7/TM1 cells, when cultured in suspension, exhibited fragmented and dense nuclei consistent with apoptosis, indicating that TM1 induces anoikis in MCF-7 cells (Figure 6a). However, there was no evidence of apoptosis in parallel suspension cultures of MCF-7 cells (Figure 6a), or adherent cultures of MCF-7 and MCF-7/TM1 cells (data not shown). Next, we investigated whether MCF-7/TM2 cells undergo anoikis. Unlike, MCF-7/TM1 cells, MCF-7/TM2 cells had exhibited resistance to anoikis, as determined by nuclear morphology (Figure 6a). Consistent with this result, parental MCF-7 and MCF-7/TM2 cells did not accumulate DNA in $subG_0$–G_1 fraction of cell cycle, which is indicative of apoptosis, and the cell cycle pattern was comparable to that of the adherent cells (data not shown). These results indicate that TM1, but not TM2, sensitize breast cancer cells to detatchment-induced apoptosis, which is in agreement with the results of anchorage-independent growth experiments (Figure 4a).

To further examine whether TM1-mediated tumor suppression of breast cancer involves anoikis or it is a cell-type-specific effect in MCF-7 cells, we tested the ability of TM1 to sensitize MDA MB 231 cells to undergo detachment-induced apoptosis. As shown in Figure 6b, MDA MB 231/TM1 cells were undergoing massive cell death (70%) by 24 h of suspension culture, while unmodified MDA MB 231 cells exhibited background apoptosis (8.3%). A likely reason for rapid induction of anoikis by TM1 in MDA MB 231 cells is that MCF-7 cells exhibit stronger cell–cell adhesion due to the expression of E-cadherin (Sommers *et al.*, 1994). Since cell–cell interactions also provide survival signals, the onset of anoikis may have been delayed in MCF-7 cells expressing TM1. Moreover, TM1 expression appears to result in increased cytoskeletal association of E-cadherin complex (Mahadev *et al.*, 2002), presumably leading to enhanced cell–cell interactions.

Since TM1 expression induces profound and rapid anoikis in MDA MB 231 cells, we next examined the kinetics of induction of anoikis by TM1 by flow cytometry, and determined the accumulation of DNA in $subG_0$–G_1 fraction of cell cycle. By 6 h of culture in suspension, TM1 expressing cells accumulated 34% of DNA in $subG_0$-G_1 fraction, (Figure 6b and c). In contrast, MDA MB 231 cells did not contain any DNA in the $subG_0$–G_1 fraction, indicating normal growth (Figure 6a). The $subG_0$–G_1 phase increased with time in suspension cultures of MDA MB 231/TM1 cells reaching 70%. MDA MB 231 cells, in contrast, exhibited normal cell cycle pattern and significantly lower (about 13%) DNA in $subG_0$–G_1 fraction compared to TM1 expressing cells by 24 h (Figure 6c). Adherent cultures of both cell types, however, contained background ($\leq 5\%$) quantities of fragmented DNA (boxes marked 'adh', Figure 6c). These data indicate that TM1 induces anoikis in breast cancer cells, and resensitization of the tumor cells to anoikis is an important mechanism through which TM1 exerts tumor suppression.

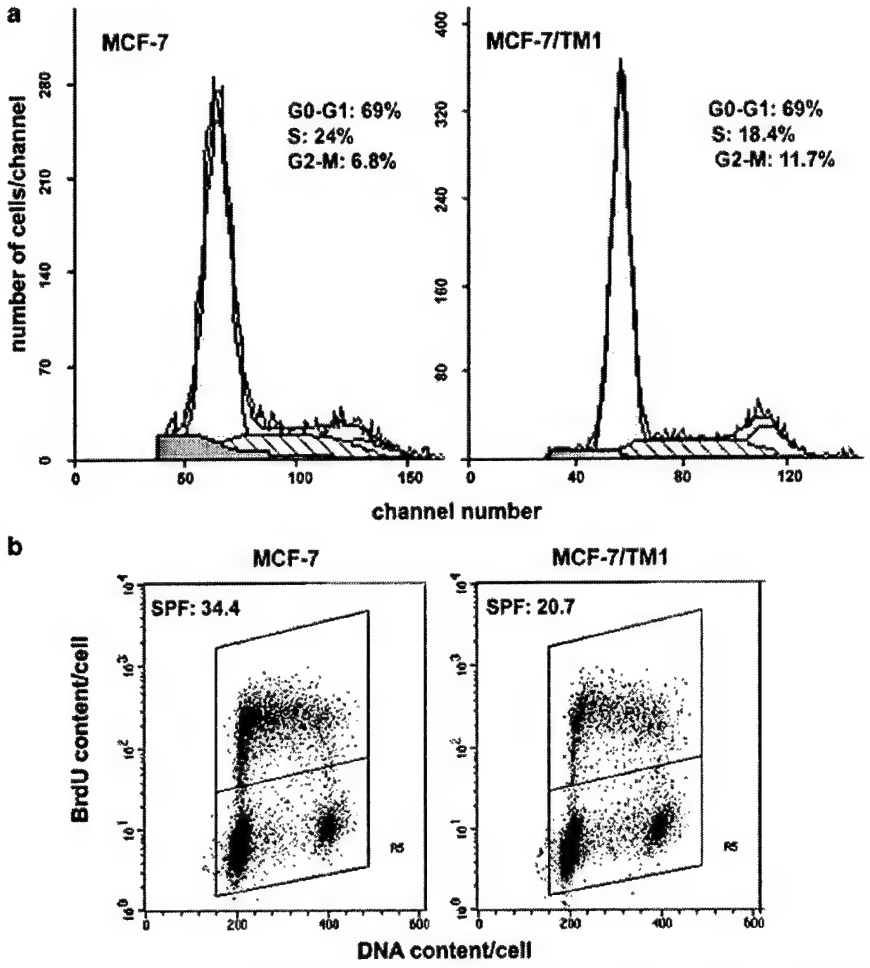

Figure 5 TM1-induced cell cycle changes MCF-7 cells. (**a**) Asynchronously growing MCF-7 and MCF-7/TM1 cells were subjected to cell cycle analysis. Trypsinized cells were detergent extracted, stained with propidium iodide and analysed by flow cytometry. Distribution of cells in each phase of the cells is given. (**b**) S-phase analysis was carried out by pulse labeling cells with BrdU as described in Materials and methods. The S-phase fraction values (SPF), indicative of the synthesis were calculated from two independent experiments. The average SPF values for MCF-7/TM1 were derived from experiments involving three independent clones

Discussion

The present work demonstrates that downregulation of TM1 expression occurs in invasive breast cancers, and that restoration of TM1 expression induces anoikis in breast cancer cells. Loss of TM1 expression is likely to destabilize microfilament architecture, and render breast tumor cells resistant to anoikis. These changes may facilitate, invasion and increased survival of tumor cells as they leave their primary locations and become malignant. Thus, suppression of TM1 may promote cytoskeletal disorganization and confer a growth advantage to the neoplastic cells.

The high-M_r TMs, including TM1, are abundantly expressed in the smooth muscle cells of the vasculature (Figures 2b and 3b) (Pittenger *et al.*, 1994). Furthermore, TM1 is expressed in fibroblasts, smooth muscle and epithelial cells (Prasad *et al.*, 1991; Pittenger *et al.*, 1994), which complicates accurate quantification of TM1 in tissues by biochemical methods. Accordingly, other researchers have reported a modest decrease in high-M_r TMs in primary breast tumors, and an increase in TM1 expression in the metastatic tumors (Franzen *et al.*, 1996, 1997). Similarly, a preliminary report indicates variable expression of TMs in prostate tumors (Ahram *et al.*, 2002). Therefore, we employed *in situ* hybridization and isoform-specific antibodies in immunohistochemistry, and have demonstrated that TM1 expression is downregulated in breast tumors.

TM1 expression, however, appears to be more variable in other common malignancies such as colon and lung cancer cell lines (data not shown). It is relevant to note that enhanced expression of either TM1, or other TMs in ras-transformed RIE cells has no effect on either cell growth or cytoskeletal organization (Shields *et al.*, 2002). Therefore, it appears that TM1 effects may

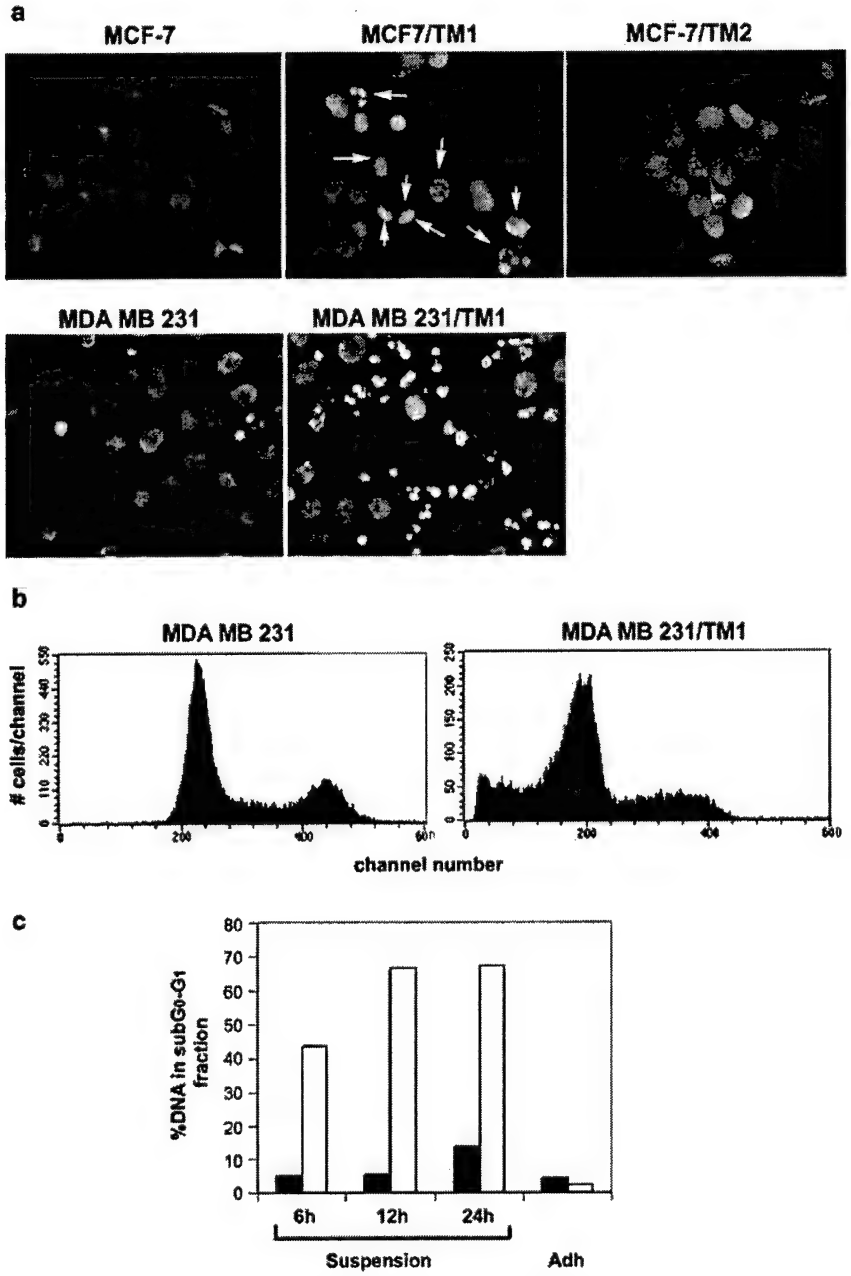

Figure 6 TM1 induces anoikis in breast cancer cells: parental MCF-7 and MDA MB 231 cells, and those expressing TM1 and TM2 were cultured on polyHEMA-coated dishes for 72 h (MCF-7-derived cells) or 24 h (MDA MB 231 derived cells). (**a**) Nuclear morphology of DAPI-stained cells is shown. Apoptotic cells are marked with arrows in MCF-7/TM1 cell panel. Cells were photographed using a fluorescent microscope with a UV filter cube at × 100 magnification. (**b** and **c**) TM1 expression induces rapid anoikis: accumulation of DNA in sub G_0–G_1 fraction, which indicates apoptosis, was measured by propidium iodide staining. MDA MB 231 and MDA MB 231/TM1 expressing cells were cultured in suspension for 6–24 h and harvested. Cell cycle distribution at 6 h is shown in (**b**). The percent DNA content in subG_0–G_1 fraction is shown (**c**). Shaded boxes are MDA MB 231 cells and the open boxes are MDA MB 231/TM1 cells. DNA content in subG_0–G_1 fraction of cells cultured under normal adhesion conditions (marked 'adh') has been quantitated. These samples do not contain any significant amount of apoptotic DNA

be tissue specific, or the transformed RIE cells may have other defects downstream of TM1, rendering TM1 ineffective.

Downregulation of TM1 appears to be one of the most common and yet remarkable changes in breast cancer. In addition to TM1, other class II tumor suppressors such as NES1 (Dhar *et al.*, 2001) and HME1 (14-3-3σ) (Ferguson *et al.*, 2000; Umbricht *et al.*, 2001) are also profoundly downregulated in a majority of breast cancers. Another actin-binding protein,

gelsolin, which severs F-actin and involved in dynamic remodeling of actin filaments, is also widely suppressed in breast tumors (Asch *et al.*, 1996; Mielnicki *et al.*, 1999).

Gross rearrangements in TM1 gene in breast tumors have not been detected (data not shown). Recent experiments with cultured breast cancer cell lines reveal that TM1 is silenced by epigenetic mechanisms involving gene methylation and histone deacetylation, which suggest that epigenetic mechanisms may regulate TM1 expression in tumors (Bharadwaj and Prasad, 2002). Thus, along with retinoic acid receptor (Sirchia *et al.*, 2000), estrogen receptor and E-cadherin (Nass *et al.*, 2000), 14-3-3σ (Ferguson *et al.*, 2000) and NES1 (Li *et al.*, 2001), TM1 gene is also turned off epigenetically during malignant transformation. It is interesting to note that while mutations in αTMs cause familial hypertrophic cardiomyopathy (Michele and Metzger, 2000) and nemaline myopathy (Donner *et al.*, 2002), the epigenetic mechanisms (Yang *et al.*, 2001) contribute to the loss of β TM gene function in neoplastic cells. However, fusions between low-M_r TMs and protein kinases such as trk receptor (Mitra *et al.*, 1987; Coulier *et al.*, 1989) and anaplastic lymphoma kinase (ALK) (Meech *et al.*, 2001) have been reported to be associated with malignant transformation. A transformation-specific low-M_r TM isoform is reported to be expressed in colon tumors, but not in normal colonic epithelia (Lin *et al.*, 2002).

While changes in the expression and activities of several key focal adhesion proteins, such as FAK (Xu *et al.*, 2000), ILK (Persad *et al.*, 2000) and Src (Coll *et al.*, 2002; Windham *et al.*, 2002), are implicated in anoikis, resensitization of breast cancer cells to anoikis by TM1, a microfilament-associated protein, is novel. Thus, TM1, like other tumor suppressors such as PTEN (Lu *et al.*, 1999), DOC/hab (Wang *et al.*, 2001) induces anoikis. Further investigations are underway to elucidate the molecular mechanisms of TM1-induced anoikis.

Materials and methods

Cell lines and reagents

Culture conditions for untransformed MCF10A, and the transformed MCF-7 and MDA MB 231 cells were previously described (Prasad *et al.*, 1992). A full-length TM1 cDNA clone (Prasad *et al.*, 1991) was subcloned into pGEM3 vector (Promega) to generate 'antisense' and 'sense probes'. The RNA probes were labeled with [^{35}S]UTP by standard techniques for *in situ* hybridization. TM1 cDNA was cloned into pAdTrack CMV plasmid and recombined with the viral backbone pAdEasy-1 vector, and transfected into HEK293 cells (He *et al.*, 1998). TM2 (Braverman *et al.*, 1996) was subcloned into pIRES2-EGFP plasmid (Clontech), and MCF-7 cells were transfected with the recombinant plasmid to coexpress GFP via an IRES sequence located downstream of the cloned TM2 cDNA.

A polyclonal pan-TM antibody that recognizes multiple TM proteins including TM1 has been previously described (Bhattacharya *et al.*, 1990; Prasad *et al.*, 1993; Mahadev *et al.*, 2002). This antibody recognizes high-M_r TMs more avidly, although it reacts with low-M_r proteins, as well. To detect TM1 specifically, we have generated several antipeptide antibodies and have determined them to be specific for TM1 (Bharadwaj and Prasad, 2002; Mahadev *et al.*, 2002). We have used one of the antibodies in immunofluorescence analyses to determine TM1 expression.

Tissue specimens

Aliquots of normal breast and tumor samples were obtained from patients undergoing surgery for their disease, through the Tumor Bank of Wake Forest University School of Medicine, Winston-Salem, NC, under the approval of the Institutional Review Board. Tissue samples (via mastectomy or lumpectomy) were collected within 60 min of surgery, fixed for 48 h in 10% phosphate-buffered formalin (pH 7.4) at room temperature, washed in 70% ethanol, embedded in paraffin, and cut into 5 μm sections for *in situ* hybridization, H&E staining and immunostaining. We have analysed a total of 25 breast tumors and their characteristics are given in Table 2.

Tissue culture cells with known TM protein profiles were trypsinized and fixed in 4% paraformaldehyde and washed in 98% ethanol followed by a brief wash with a mixture of 98% ethanol and ether (1 : 1 v/v). The cell pellet was dried and embedded in paraffin. Sections (5 μm) were cut and used in *in situ* hybridization and immunofluorescence experiments. These sections were used to test and validate the screening methods.

In situ *hybridization*

To detect TM1 mRNA, *in situ* hybridization was performed using a method described by Wilcox *et al.* with some modifications (http://www.emory.edu/WILCOX). The formalin-fixed, paraffin-embedded tissue sections were deparafinized and hydrated, and processed for *in situ* hybridization as previously described (Mondy *et al.*, 1997). For quantitation of TM1 mRNA signal, nonoverlaping images were imported into an image analysis program essentially as described (Mondy *et al.*, 1997). Corresponding areas from parallel sections probed for nonspecific hybridization were subtracted. Results from each sample were averaged, and comparisons were made between the tumor and normal area.

Immunocytochemical analysis of TM expression in breast tumors

Deparaffinized tissue specimens were employed for immunofluorescence using TM1-specific antibody or pan-TM-antibody as the primary antibody. Serial 5-μm-thick tissue sections were subjected to immunofluorescence staining for detection of TM1, or multiple TMs as described previously (Shah *et al.*, 2001; Mahadev *et al.*, 2002). For control purposes, samples were processed in parallel except with the omission of the primary antibody. Anti-rabbit immunoglobulin antibody conjugated with Texas Red was used as the secondary

Table 2 Breast tissues used to analyse TM expression

Total tissues analysed—25
Age range 35–87years, median age 54 years
Race: 24 w/f; 2 b/f
Ductal carcinoma – 21
Lobular carcinoma – 2
Phyllodes tumor – 1
Not determined from the initial pathology reports (unknown) – 1
Adjacent normal tissue – 24

antibody (Molecular Probes) followed by counterstaining with 10 ng/ml DAPI (in methanol w/v) to visualize the nuclei and to identify the tissue architecture. Microscopic analysis was performed using a ×25 Neofluar oil objective. Microscopic fields were captured with a Zeiss Axiocam camera, keeping the gain and exposure constant. Autofluorescence of tissue sections was observed using green fluorescence (FITC) channel, which did not interfere with the specific signal. TM1 expression was determined by quantifying the luminosity of the images using Adobe Photoshop (version 6.0) with the magic wand tool and the histogram function.

Cell cycle and anoikis experiments

Cell cycle analyses using propidium iodide and BrdU were carried out as described (Darzynkiewicz *et al.*, 2001). Briefly, cells were labeled for 1 h with BrdU (Sigma) at 20 μM concentration, harvested and fixed. Cells were reacted with anti-BrdU antibody (Becton-Dickinson) followed by incubation with FITC-conjugated second antibody (Sigma). Finally, the samples were stained with 20 μg/ml propidium iodide and subjected to flow cytometric analysis. S-phase fraction was calculated as a percentage of Brdu containing cells in the total cells (combined fractions of propidium iodide and BrdU containing cells).

Anoikis experiments were carried out on polyHEMA (Sigma) coated dishes (final concentration 10 μg/ml, w/v in ethanol, two applications) as described by Zhu *et al.* (2001). Cells were cultured in serum-free, 1% BSA containing medium. Cells were harvested and collected by Cyospin, and stained with 10 ng/ml DAPI to visualize nuclei. The cells were photographed using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany) with a ×100 objective, and a Zeiss Axiocam camera. Images were imported into Adobe Photoshop. To calculate the percent cells undergoing anoikis, several image fields were photographed and the percent apoptotic nuclei were counted. The DNA content in subG_0–G_1 fraction was calculated as flow cytometry (Darzynkiewicz *et al.*, 2001).

Other methods

Northern blot analysis was used to quantify TM1 mRNA expression (Mahadev *et al.*, 2002). For protein analyses, cultured cells were solubilized in lysis buffer (Mahadev *et al.*, 2002). The membranes were probed with TM1-specific antibody or pan-TM antibody and α-tubulin antibody (Bharadwaj and Prasad, 2002; Mahadev *et al.*, 2002). Anchorage-independent growth was measured by plating 2×10^4 cells on soft agar plates and culturing for 2–3 weeks. In experiments involving adenoviral infections, cells were infected for 24 h and then plated on soft agar. To transduce TM1, the recombinant virus at 180 MOI was used and the control virus was used at 100 MOI to achieve about 50% infectivity of MCF-7 cells, as measured by GFP expression. Statistical analyses were performed using PROC MIXED within the Statistical Analysis System for personal computers (SAS Institute, Cary, NC, USA).

Acknowledgements
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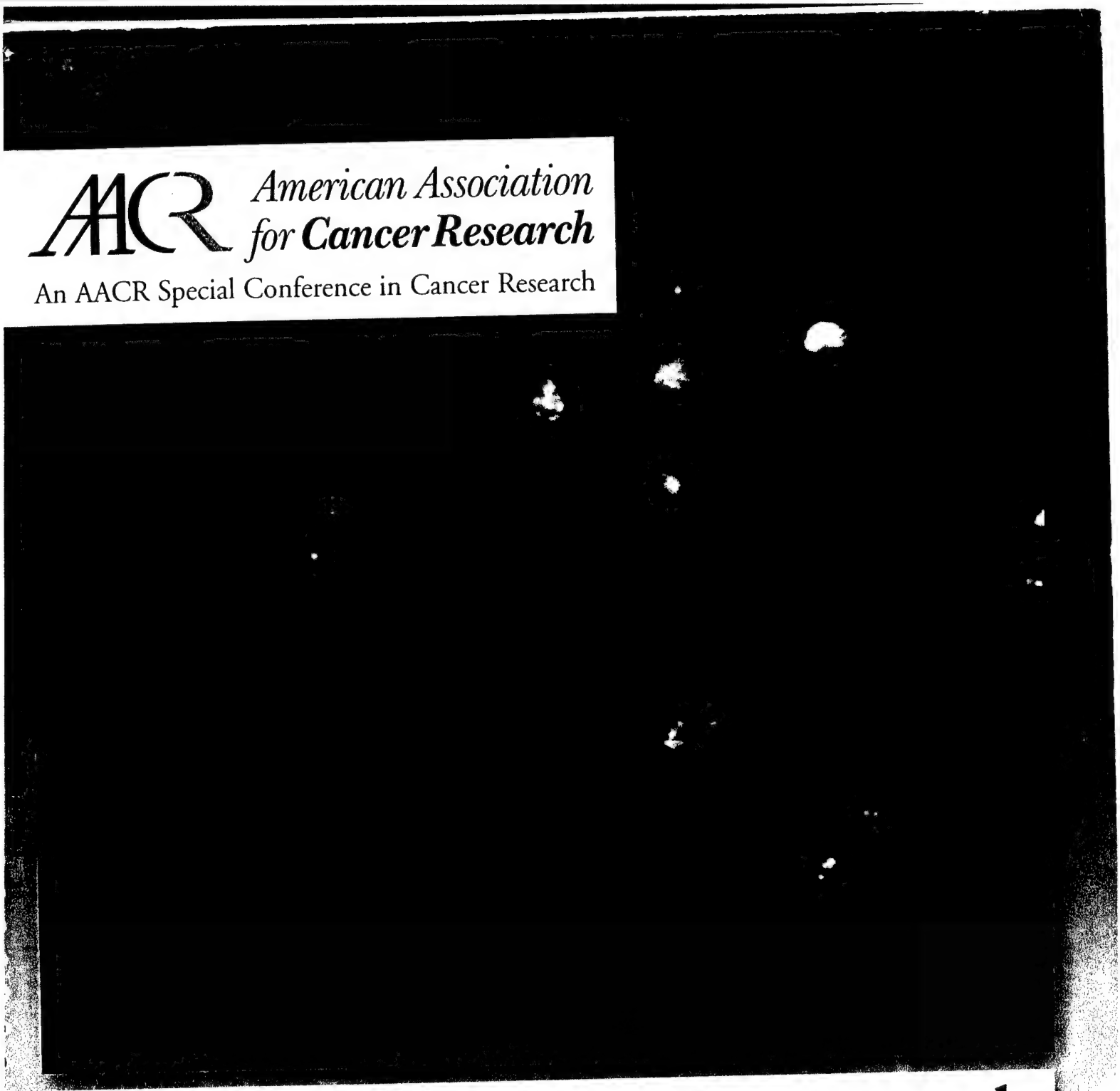

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Cytoskeletal Proteins as Regulators of Breast Cancer: Anoikis and Tumor Suppression by Tropomyosin-1, a Microfilament Stabilizing Protein

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While surgery remains as the most effective means to control primary breast cancer, most fatalities in breast cancer patients occur due to the metastatic disease. Two most common properties of malignant cells are the presence of disorganized cytoskeleton and the ability to grow at non-physiological loci. For example, it is well established that cytoskeleton regulates cell morphology, cell division and cell motility. Cytoskeletal disorganization, caused by changes in the expression of cytoskeletal proteins during neoplastic transformation, leads to deregulation of these processes and facilitates invasion and malignant growth. Second, normal cells undergo anoikis (detachment-induced apoptosis) when removed from extracellular matrix (ECM). In contrast, tumor cells are resistant to anoikis and proliferate in the absence of adhesion-derived survival signals, as metastases. Therefore, investigations into mechanisms that promote metastasis may lead to better options to treat breast cancer. Earlier work from this laboratory has identified the loss of expression of tropomyosin isoform 1 (TM1)¾a microfilament stabilizing protein¾ as a common biochemical change in the breast cancer cell lines. Consistent downregulation of TM1 appears to be specific for breast cancer cells, as about 50% lung and colon carcinoma cells do contain significant levels of TM1. TM1 expression is extinguished in breast cancer cells by epigenetic mechanisms involving histone deacetylation and gene methylation. Restoration of expression of TM1 in two widely studied breast cancer cell lines results in reorganization of actin cytoskeleton. Significantly TM1 also inhibits the malignant growth of breast cancer cell lines, independent of p53 or estrogen receptor status. The anti-neoplastic effects of TM1 are isoform specific; other closely related TMs do not affect the malignant growth properties of breast cancer cells. Collectively these results suggest that suppression of TM1 may be a key event during mammary carcinogenesis, and that TM1 is a class II tumor suppressor. To investigate the significance of the lack of TM1 expression in the established breast cancer cell lines, we have assessed changes in TM1 expression in 25 normal and 25 breast tumor tissue specimens collected at the Wake Forest University School of Medicine. By *in situ* hybridization and immunofluorescence, TM1 expression is readily detectable in the normal ductal epithelium, but undetectable in the tumor tissues. These results, for the first time, demonstrate that the loss of TM1 expression is a common biochemical event in breast cancer. Investigations into TM1-induced tumor suppression suggest that TM1 induces anoikis in breast cancer cells. While several proteins such as integrins and focal adhesion kinases are known to regulate adhesion-dependent survival, modulation of anoikis by a structural, microfilament protein is novel. Thus, the loss of TM1 expression during the neoplastic transformation contributes to loss of cellular morphology, and may confer resistance to anoikis. Ongoing work addresses the detailed mechanism of TM1-induced anoikis. Because TM1 suppresses the basic mechanisms that are essential for metastasis, it is likely these investigations may identify novel pathways/targets amenable for drug development.

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Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells

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Abstract

Tropomyosins (TMs) are a family of microfilament binding proteins, which are suppressed in the transformed cells. We have investigated the mechanism of suppression of TMs, in particular that of tropomyosin-1 (TM1), in breast cancer cells. Inhibition of DNA methyl transferase with 5-aza-2′-deoxycytidine (AZA) alone did not induce TM1 expression. However, combined treatment of trichostatin A (TSA) and AZA resulted in readily detectable expression of TM1, but not that of other TM isoforms. Upregulation of TM1 expression paralleled with the reemergence of TM1 containing microfilaments, and in abolition of anchorage-independent growth. The synergistic action of AZA and TSA in reactivation of TM1 gene was also evident in ras-transformed fibroblasts. These data, for the first time, show that hypermethylation of TM1 gene and chromatin remodeling are the predominant mechanisms by which TM1 expression is downregulated in breast cancer cells.

Keywords: Tropomyosin; Methylation; Histone acetylation

1. Introduction

Tropomyosins (TMs) are important actin binding proteins that stabilize microfilaments from the action of gel severing proteins [1,2]. Non-muscle cells elaborate multiple isoforms of TMs, some of which are expressed with a high degree of tissue specificity by alternate splicing. TMs are generally grouped into high (284 amino acids) and low (248 amino acids) M_r species, and they share significant sequence homology among the individual isoforms and across the species. Nevertheless, TMs exhibit substantial diversity in terms of their size, binding affinity to actin, subcellular localization and in interactions with other proteins, thus suggesting that individual TMs may differ in their biological functions. For example, TM1, a high M_r TM isoform with 284 amino acids, is expressed in fibroblasts and epithelial cells, but not in skeletal muscle tissue. TM1 has been shown to bind to actin with a higher affinity, and its interactions with actin are preferentially promoted by other TM isoforms, or other actin binding proteins such as caldesmon.

The expression of high M_r TMs is downregulated in transformed cells [1,2]. Two reports indicate that downregulation of TMs is rapid and precedes the morphological transformation of growth factor induced transformation [3,4]. Studies on the mechanism of downregulation of tropomyosins in fibroblasts suggested that both MEK-dependent and -independent signaling pathways are involved [5,6].

Previous work from this laboratory has shown that

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in breast cancer cells, multiple TM isoforms are suppressed [7]. Additionally, the expression of TM1 was consistently lost in breast cancer cells, indicating that loss of TM1 expression could be a common biochemical event in mammary carcinogenesis. Further investigations revealed that restoration of TM1 suppresses the malignant phenotype of oncogene-transformed cells and breast cancer cells, suggesting that TM1 is a general suppressor of neoplastic growth phenotype [8–10]. The focus of the present work is to elucidate the mechanism of downregulation of TM1 in breast cancer cells. Several important genes involved in a wide range of pivotal cellular processes are transcriptionally silenced by gene methylation in breast cancer [11]. Therefore, we examined the role of promoter methylation of TM1 gene as a possible mechanism in the loss of TM1 expression in cancer cells.

2. Materials and methods

Cell culture and drug treatments: MCF-7 and MDA MB231 cells were purchased from ATCC, and the sources and culture conditions of MCF10A and DT cells have been previously described [7,12]. 5-Aza-2′-deoxycytidine (AZA) was added to the subconfluent cultures at a final concentration of 8.8 μM. For the experiments involving combined treatment of AZA and TSA, TSA was added to the culture medium at 300 nM after pretreating the cells with AZA for 24 h. The cells were routinely harvested as indicated.

RNA isolation and northern blotting for the expression of TMs was performed as described elsewhere [12]. For detection of TM1, a full length cDNA encoding TM1 was used and the membranes were washed at high stringency (0.1 × SSC at 65°C). To detect the low M_r TM isoforms TM4 (3.0 kb mRNA) and TM5 (2.3 kb mRNA), probes M1401 and M29, respectively, were used at the high stringency conditions [13,14]. We also used a probe, M1558, which recognizes TM1 and other muscle-type TM mRNAs. The northern blots were routinely stripped and reprobed with β-actin or GAPDH probes for load controls. The signals were quantified by a Molecular Dynamics phoshor imager (Typhoon 8600), and the ratios obtained with a specific probe and β-actin (or GAPDH) were calculated. These values represent a quantitative measure of the changes in the gene expression.

Cell lysates were prepared using Tris buffer containing 1% NP-40, 0.5% deoxycholate and a cocktail of protease inhibitors [12]. Two different antibodies were used to assess TM1 expression: a polyclonal antibody that recognizes multiple TM isoforms [7,12]; and, a TM1-specific antibody. A 45 amino acid region of TM1 (171–215 aa; [15]) was used to generate an anti TM1-specific antibody in rabbits. This antibody specifically recognizes TM1, but shows little or no reactivity against other TM isoforms. Fifty micrograms of cellular proteins were routinely analyzed in western blotting experiments. The blots were stripped and reprobed with anti α-tubulin antibody for load controls. After the chemiluminescence reaction, the exposed X-ray films were scanned and the signals were quantitated on the Typhoon imager. The ratios of the signal obtained with TM antibody and α-tubulin were used as a measure of changes in gene expression.

Immunofluorescence microscopy was performed essentially as described previously, using a TM antibody that recognizes multiple TM proteins [12]. Anchorage-independent growth assays were performed in soft agar according to the previously published procedures, except with the addition of AZA and TSA [8,10]. The drugs were added to the top agar at the concentrations indicated above, and the cultures were fed with or without (control) the appropriate drugs every 48 h. The samples were viewed with Olympus BX 60 microscope at 4× magnification (objective), and the individual colonies were enumerated.

The potential methylation islands on the TM1 gene were identified by GrailEXP v3.2 program (http://compbio.ornl.gov/grailexp).

3. Results and discussion

3.1. Isolation of TM1 gene

In breast carcinoma cells, TM1 expression is completely and consistently abolished. To investigate the mechanism of inhibition of TM1 expression, human TM1 gene was isolated by polymerase chain reaction (PCR) screening of a BAC library. A single BAC clone was isolated and the presence of TM1

gene was confirmed by exon specific PCR, restriction digestion mapping and partial sequencing. The BAC clone contained the entire TM1 gene consisting of 11 exons. TM1 is generated by alternate splicing from exons 1–6, 8, 9 and 11 [16]. The sequence information obtained was in agreement with the data deposited in GenBank (accession numbers: AF209746 and AL133410), and therefore, is not shown.

Computer analysis of the 5′ untranslated region (5′UTR) indicated the presence of three potential locations for methylation of cytosines residues in the C_PG islands: −1252 to −861; −712 to −433; and, −204 to +117, with 'A' of initiation codon numbered as +1. These islands span a 1369 bp region in the 5′UTR through the first exon. Since the epigenetic silencing of many key genes is effected by promoter hypermethylation in cancers, including those originating in breast, we have examined whether promoter hypermethylation accounts for the loss of TM1 expression in breast cancer cells [11,17].

3.2. Hypermethylation and histone deacetylation of TM1 gene in cancer cells

Transcriptional silencing of many genes occurs during development and disease conditions, including in cancer. Gene methylation and chromatin remodeling allow reversible activation and inactivation of target genes both permanently as well as transiently [17]. Inactivation of the key regulatory genes either by mutation and/or epigenetic regulation results in the same biological consequence in that both processes disrupt normal regulatory circuits. Epigenetic silencing of many classical tumor suppressor genes such as APC, BRCA1, E-cadherin and Rb occurs in sporadic cancers, suggesting that hypermethylation is not a random event. Furthermore, many genes that are not fully documented as tumor suppressors, or important regulators, are inactivated by hypermethylation. It is likely that epigenetic regulation may be the primary mode of inactivation for some regulatory genes [17]. For example, HME1 (14-3-3σ) appears to be downregulated in breast tumor by promoter methylation [18].

Normal mammary epithelial cells express seven different TMs, among which TM1 is a prominent isoform that is also found in fibroblasts [7]. In breast cancer cells, TM1 expression is abolished, while the expression of other TMs varies. MCF-7 cells lack TM1, TM38 and TM2 isoforms, as shown by two-dimensional gel analyses [7]. We have used MCF10A cells as controls for normal mammary epithelial cells. It should be noted that MCF10A and MCF-7 are not isogenic cell lines, and therefore, only a qualitative comparison of TM expression profiles between the cell types is possible (Kalyankar et al., manuscript submitted). To investigate the mechanism of TM1 gene silencing, MCF-7 cells were treated with an inhibitor of DNA methyl transferase (DNMT), 5-aza-2′deoxycytidine (AZA) for 24 h, and RNA and protein were analyzed for TM1 expression, as described in Section 2. MCF-7 cells lack TM1 mRNA and protein (Fig. 1A,B). Addition of AZA to the cultures resulted in a modest induction of TM1-specific 1.1 kb mRNA and TM1 protein. The level of expression of TM1 attained with AZA alone was only barely detectable over the background. Prolonged culture with AZA for up to 9 days did not result in further enhancement of TM1 levels (data not shown).

Histones, in acetylated state, cause relaxation of tightly supercoiled chromatin leading to improved accessibility of DNA binding proteins and transcription factors to promoter region, culminating in gene transcription [19,20]. Histone deacetylases promote condensation of chromatin and gene silencing. Therefore, we examined whether TM1 expression is repressed by this mechanism. In fact, several genes, including estrogen receptor α and gelsolin are regulated by this mechanism [21,22]. Furthermore, inhibition of histone deacetylase (HDAC) activity suppresses the growth of breast cancer cells [23,24]. MCF-7 cells were incubated with trichostatin A (TSA), a well characterized inhibitor of histone deacetylation for 24 h and analyzed for TM1 expression. TSA treatment did result in a very small increase in TM1 expression (Fig. 1). Extended culture of MCF-7 cells with TSA alone up to 72 h did not induce TM1 to any higher levels (data not shown).

We next evaluated the combined effect of inhibition of DNMT and HDAC on TM1 gene expression. It has been postulated that gene methylation and histone deacetylation act as two layers in ensuring strong silencing of certain important genes in tumor development, and FMR1 gene which is mutated in fragile X syndrome [17,25,26]. Consistent with this notion, combined treatment of AZA and TSA resulted in robust reactivation of genes such as hMLH1,

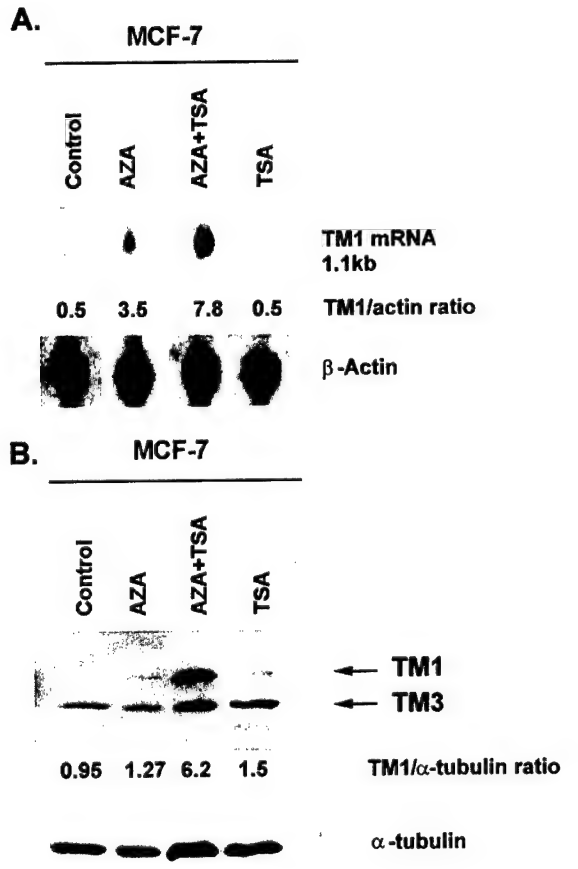

Fig. 1. Induction of TM1 by AZA and TSA. (A) Total RNA from the control MCF-7, AZA, TSA and AZA + TSA treated MCF-7 cells was probed for TM1 mRNA expression by a full length TM1 cDNA. For load controls, the same membrane was reprobed with β-actin probe. The ratio of TM1/β-actin signal is given. Control MCF-7 cells lack TM1 mRNA and therefore, the value indicates that of a background signal. (B) Immunoblotting of TM1 protein. Cellular extracts were analyzed by western blotting with a TM antibody. The positions of TM1 and TM3 are indicated. For load controls, the membrane was reprobed with anti-α-tubulin antibody. MCF-7 cells lack TM1 and the values represent background. TM1 is induced by a combination of AZA and TSA.

CDKN2A, TIMP3 and FMR1 [25,26]. Therefore, we tested whether AZA and TSA together would upregulate TM1 expression. Incubation of MCF-7 cells with AZA for 24 h followed by AZA and TSA for another 24 h (AZA total for 48 h) synergistically enhanced the expression of TM1 mRNA and the protein (Fig. 1A,B, respectively). This finding indicates that hypermethylation and chromosomal compaction by histone deacetylation are major mechanisms by which TM1 expression is silenced in breast cancer cells.

To investigate whether AZA and TSA mediated induction is specific to TM1, or the expression of all the TMs are generally upregulated by these drugs, mRNA levels of two low M_r TMs, were assessed. We utilized a cDNA probe, M1558 that is known to hybridize with several high M_r TMs to monitor the changes in the expression of TM1, TM2 and TM3 [13]. This probe showed the reactivation of TM1 gene in the cells treated with AZA and TSA together, but not other TMs. The results obtained with M1558 probe are identical to those described in Fig. 1A, and therefore, the data are not shown. Under the conditions of TM1 induction in MCF-7 cells, no significant change in the expression of TM4 and TM5 was detected by the drug treatment (data not shown). These data indicate that expression of TM genes is differently regulated, and that only TM1 expression is inactivated by gene methylation in breast cancer cells. Previous reports indicate that the changes in the expression of low M_r TMs is not often downregulated in cancer cells, and in some instances they are upregulated to compensate the loss of high M_r TMs [27].

3.3. Induced TM1 participates in microfilament assembly

TM1 is an important actin binding protein. Previous work from this laboratory showed that enhanced expression of TM1 results in microfilament reorganization in ras- and src-transformed fibroblasts, and in MCF-7 breast carcinoma cells [8–10]. To test whether the induction of TM1 expression by AZA and TSA would result in the incorporation of TM1 in microfilaments, immunofluorescence microscopy was performed (Fig. 2). The control MCF-7 cells lack the expression of TM1 and other TMs, and do not exhibit well-developed TM-containing microfilaments (panel a). However, MCF-7 cells do contain prominent actin filaments (panel b), possibly consisting of TM3, and other low M_r TMs (panel c). Similar results (data not shown) were obtained when the cells were treated with either AZA or TSA alone. This finding is consistent with the immunoblotting data which show a small increment in TM1 expression

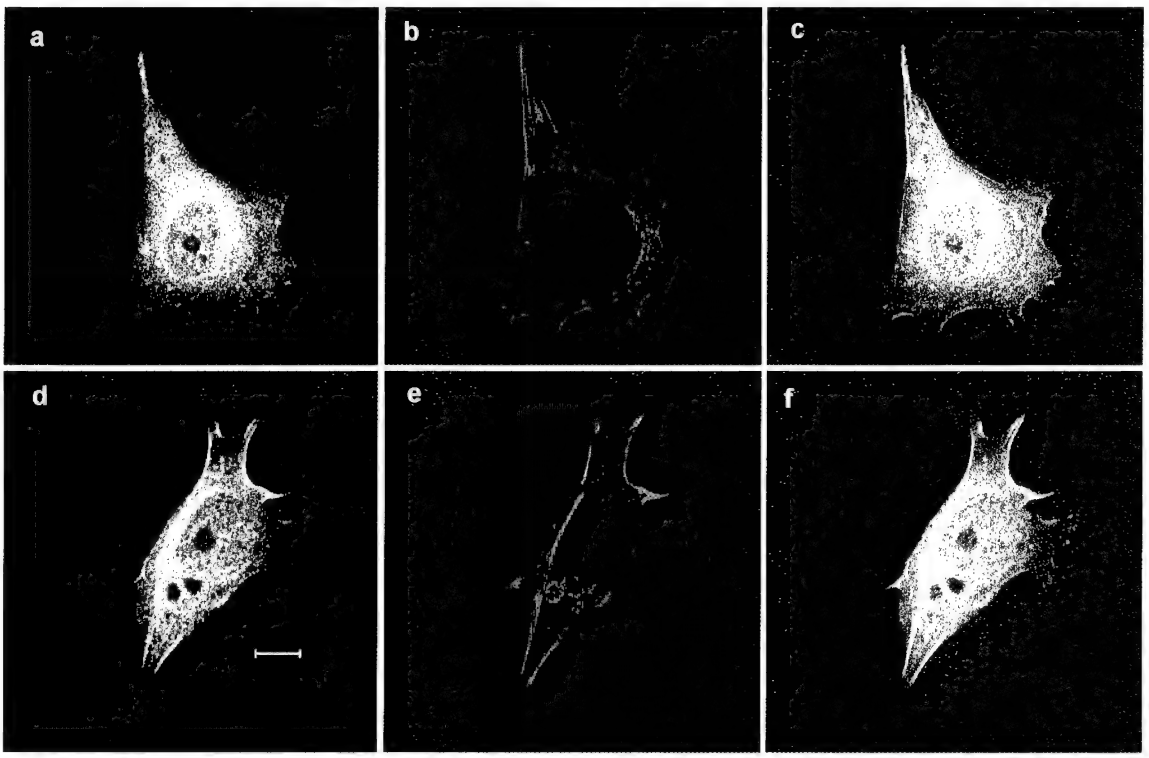

Fig. 2. Reorganization of microfilaments by induced TM1: control MCF-7 cells (panels a–c) and those treated by AZA and TSA (panels d–f) were stained with anti-TM antibody (a, d), or phalloidin (b, e). The merged images are shown in panels c and f. The drug treatment induces TM1 expression, and the induced TM1 participates in microfilament reorganization, as evident from d–f. The sizing bar indicates 10 μm.

with either AZA or TSA. When the cells were primed with AZA, and treated with TSA, the presence of TM1 containing microfilaments were clearly evident (panel d), and TM1 was colocalized with actin in those filaments (panels e and f).

3.4. Suppression of the transformed growth by AZA and TSA

Data from this laboratory show that TM1 is a suppressor of the malignant growth phenotype [8–10]. To determine whether AZA and TSA inhibit the anchorage-independent growth – a hallmark of the neoplastic growth, MCF-7 cells were cultured with AZA, TSA and together with AZA and TSA in soft agar. Control MCF-7 cells grew rapidly and formed colonies in soft agar (Fig. 3). The presence of AZA suppressed the colony formation appreciably (76%), while TSA alone was marginally (9.4%) effective. These data indicate that alterations in the expression of other key genes by AZA may contribute to the diminished growth in agar. In fact, key genes regulating growth such as retinoic acid receptor β2 and glutathione transferase P1 gene are activated by AZA treatment of MCF-7 cells [11]. Furthermore, methylation profiling of genes revealed that several genes are regulated by promoter methylation in breast cancer [28,29]. Reactivation of these genes or the tumor suppressor genes may be responsible for suppression of soft agar growth of AZA-treated MCF-7 cells. A more pronounced suppression (82%) in the anchorage-independent growth was accomplished in the presence of both AZA and TSA, confirming the synergistic action of these two drugs. A recent report [30] indicating that AZA and TSA synergistically upregulate estrogen receptor α gene and retinoic acid receptor β, and kill MDA MB231 cells, is in agreement with our finding that a combination of these two drugs is effective in abolishing the anchorage-independent growth.

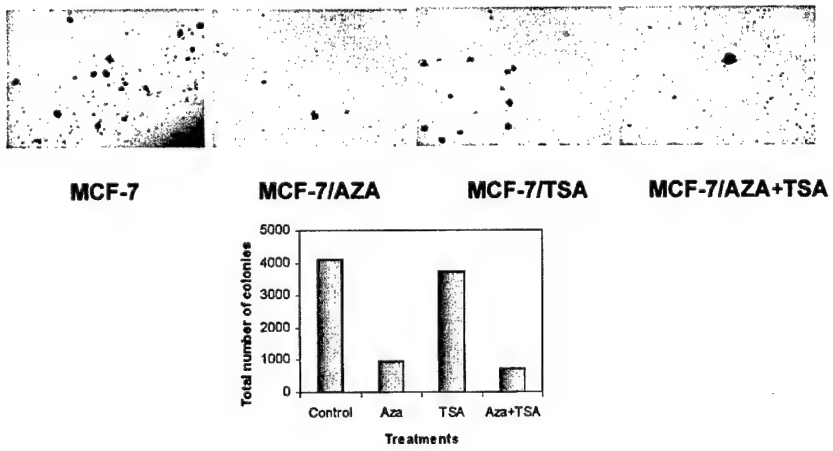

Fig. 3. Effect of AZA and TSA on the anchorage-independent growth. MCF-7 cells were cultured with either AZA, TSA and AZA + TSA, and counted, as described in Section 2. Photomicrographs of culture plates and the quantitative data are shown. Although AZA treatment decreases colony formation, the combined treatment of AZA and TSA is more effective in suppressing anchorage-independent growth.

3.5. Induction of TM1 expression in other cancer cells

To investigate whether gene hypermethylation and histone deacetylation account for the silencing of TM1 gene in other malignantly transformed cells, we assessed TM1 expression in two different cell lines. In MDA MB231 breast cancer cells, the 1.1 kb mRNA that codes for TM1 protein is lacking (Fig. 4A). As observed with MCF-7 cells, only the combined treatment of AZA and TSA reactivated the expression of TM1 mRNA. MDA MB231 cells also express an epithelial cell-specific TM isoform, TM38, which comigrates with TM1 (Fig. 4B, bottom panel) [7]. Therefore, an antipeptide antibody that recognizes TM1 with a high degree of specificity was used to assess TM1 expression in this cell line (Fig. 4B, top panel). Immunoblotting with this antipeptide antibody reveals the presence of TM1 in MCF10A cells, but not in MDA MB231 cells.

Treatment of cells with either AZA or TSA did not result in the restoration of TM1 mRNA or protein levels (Fig. 4A,B). Pretreatment of MDA MB231 cells with AZA, followed by addition of AZA and TSA, produced a significant induction of TM1, suggesting that inhibition of DNMT and HDAC are required for reactivation of TM1 expression. However, pretreatment of either of the breast cancer cell lines with TSA followed by addition of AZA did not upregulate the expression of TM1 (data not shown), supporting the notion that prior demethylation of TM1 gene may be a requirement for the chromatin decompaction by acetylated histones, and ultimately TM1 gene transcription [25]. Correspondingly, TM1 protein was detected in the cell lysates of the cultures treated with AZA and TSA (Fig. 4B, top panel). As observed with MCF-7 cells, culturing MDA MB231 cells with either of the drugs, or together did not result in significant changes in the expression of TM4 and TM5 (data not shown).

DT cells are ki-ras transformed NIH3T3 cells which are extensively used in this laboratory to study the role of cytoskeletal proteins in the cellular transformation [31]. The DT cells express TM1 at $\geq$50% of the levels found in NIH3T3 cells, and essentially undetectable levels of TM2 and TM3 [8,31]. In DT cells, TM1 RNA expression is downregulated to 50% levels found in NIH3T3 cells (Fig. 5A). In contrast to the breast cancer cells, AZA treatment produced a two-fold enhancement of TM1 mRNA, as evident from TM1/GAPDH ratios. TSA treatment, on the other hand, was less effective in upregulating TM1 mRNA. Once again, the combined treatment of AZA and TSA resulted in a further upregulation of TM1 mRNA ($\geq$three-fold).

TM1 protein levels in DT cells are suppressed by about 65%, as measured by TM1/α-tubulin ratios, when compared to normal NIH3T3 cells (Fig. 5B), exceeding the 50% decrease in mRNA levels. AZA

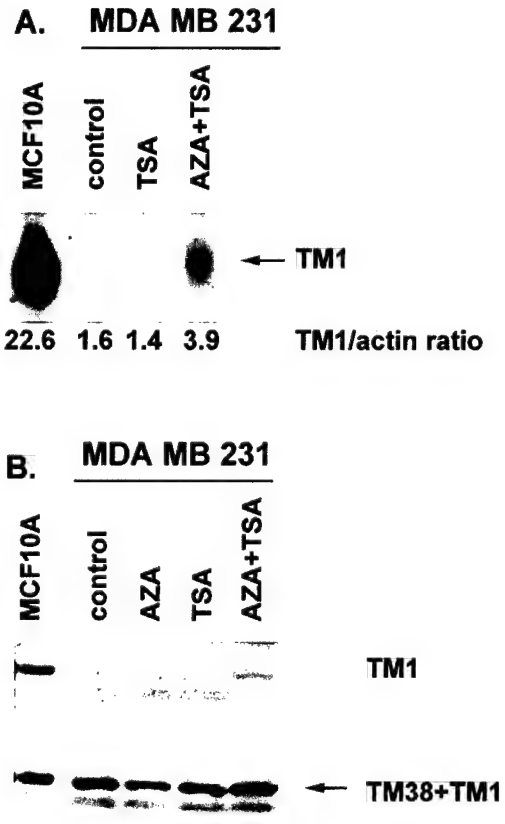

Fig. 4. Induction of TM1 in MDA MB231 cells by combined treatment of AZA and TSA. (A) Northern blotting. For comparison, RNA isolated from MCF10A cells is included. Control MDA MB 231 cells do not express TM1. (B) Immunoblotting of drug treated cellular lysates for TM1 expression. The MDA MB231 cells express an epithelial specific TM isoform, TM38, which comigrates with TM1. The polyspecific TM antiserum detects TM38 and TM1, and therefore the signal is present in both control and treated MDA MB231 cells (bottom panel). MCF10A cells express both TM1 and TM38. A TM1-specific antibody recognizes only TM1, but not TM38. Therefore, a signal is detected in MCF10A cells, but not in untreated MDA MB231 cells, or those treated with either AZA or TSA (top panel). TM1 expression, however, is induced by the combined treatment of AZA and TSA.

treatment of DT cells resulted in about 40% increase in TM1 expression compared to control DT cells. In addition, TSA treatment also enhanced TM1 expression to a much lesser, but to a detectable degree (12%). Combined treatment of DT cells with AZA and TSA produced further enhancement in TM1 protein levels (53% increase) compared to the control DT cells, underscoring the role of promoter methylation and chromatin conformation in regulating TM1 gene expression in tumor cells.

In addition to TM1, another high M_r TM isoform, TM2 was reactivated when treated with AZA and TSA (Fig. 5B). However, TM2 expression was substantially lower than in NIH3T3 cells. Reactivation of TM2 expression was not observed in MCF-7, or MDA MB231 cells, pointing to the differences in the regulation of TM proteins in different cancer cells. Thus, while TMβ gene is generally inactivated by gene methylation in cancer cells, it appears that down-regulation of TMα gene (which codes for TM2 and TM3) via promoter methylation may occur more specifically in ras transformation.

TM1 was suggested to belong to the class II tumor suppressors, which are transcriptionally silenced during the malignant transformation of cells [32]. Data presented in this communication indicate that promoter hypermethylation and chromatin remodeling by histones are primarily involved in silencing of TM1 gene in the malignant cells. However, there appear to be additional posttranscriptional mechanisms governing the expression of TM1, as evident from Fig. 5.

As discussed above, loss of TM1 expression is a common feature of many different malignant cells. Suppression of high M_r TMs, in particular that of TM1 and TM2 occur very rapidly, before the morphological transformation is evident, suggesting that the loss of TM1 may be an early event during tumorigenesis. Therefore, the loss of TM1 expression may serve as a potential biomarker. However, because of the high degree of sequence homology among the TMs, and a high degree of expression in the stromal and smooth muscle components of the tissue block has rendered the assessment of TM1 in human tumors difficult. Therefore, determination of the methylation status of TM1 gene may be useful in developing an approach to analyze and utilize TM1 expression as a prognostic indicator. Furthermore, since TM1 is a suppressor of the malignant growth, the induction of TM1 protein may contribute to the antineoplastic properties of AZA and TSA.

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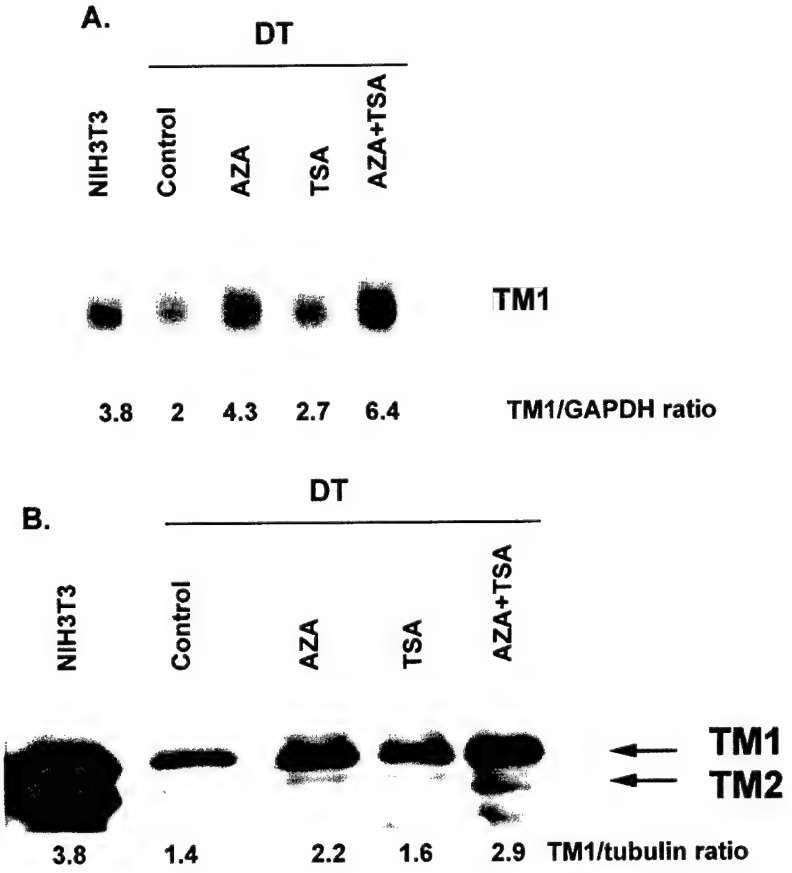

Fig. 5. Induction of TM1 expression in ras-transformed fibroblasts: (A) Northern blotting for TM1 expression. Total RNAs from NIH3T3, DT (ras-transformed NIH3T3 cells), along with DT cells treated with the drugs were isolated and probed with a full length TM1. The ratios of TM1/GAPDH signals are given. TM1 expression is induced significantly by AZA or AZA and TSA treatment. (B) Induction of TM proteins by AZA and TSA: cell lysates were immunoblotted using a polyspecific TM antibody. Treatment of DT cells with AZA significantly induced TM1 expression. However, the combination of AZA and TSA synergistically enhanced TM1 expression, and also reactivated TM2.

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Suppression of the Transformed Phenotype of Breast Cancer by Tropomyosin-1

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Changes in the expression of microfilament-associated proteins, such as tropomyosins (TMs), are commonly found in malignantly transformed cells. Previous work from this laboratory has shown that tropomyosin-1 (TM1) expression is consistently abolished in human breast carcinoma cell lines, suggesting that the loss of TM1 could be a common biochemical event in the transformation of mammary epithelium. To investigate whether changes in TM1 expression are causally linked to mammary carcinogenesis, we have tested the hypothesis that TM1 is a tumor suppressor of breast cancer. MCF-7 cells, which lack TM1, were utilized as a model of human breast cancer and transduced to reexpress TM1 protein. Restoration of TM1 expression in MCF-7 cells (MCF-7/T cells) resulted in a slower growth rate, but cells remained sensitive to growth control by estrogen. TM1 expression in MCF-7 cells resulted in the emergence of TM-containing microfilaments. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. TM1 reexpression alters the interaction of the E-cadherin–catenin complex with the cytoskeleton, indicating that TM1-induced cytoskeleton could play a significant role in suppression of the malignant phenotype. Taken together with our previous work on transformed murine fibroblasts, the results presented in this communication indicate that in nonmuscle cells TM1 functions as a suppressor of transformation.

Key Words: **tropomyosin; breast cancer; cytoskeleton; E-cadherin; β-catenin; tumor suppression.**

INTRODUCTION

During the neoplastic transformation, cells accumulate several different mutations and undergo extensive changes in gene expression. While the etiology of the vast majority of tumors is unknown, it is generally accepted that multiple genetic events contribute to the neoplastic transformation of cells [1]. Some of the changes include loss of tumor suppressor genes, activation of cellular proto-oncogenes, and inactivation/deregulation of the function or expression of key intracellular proteins. The mortality due to cancer is primarily a consequence of the ability of neoplastic cells to invade and metastasize in tissues where the cells do not normally grow. Tumor metastasis involves multiple steps including the loss of normal growth controls, the derangement of cytoskeletal organization, and the capacity to become motile and invasive [2–4]. It has been postulated that deregulation of growth factor (serum)-controlled and integrin-regulated adhesion pathways together contribute to the complete transformation involving accelerated proliferation and anchorage-independent growth [5]. In addition, tumor cells also manifest altered cell–cell adhesion and abnormal microfilaments, which facilitate invasion. Microfilaments are linked to both integrin and cadherin–catenin complexes which regulate cell–matrix and cell–cell adhesion, respectively.

While tropomyosins (TMs) have been known to function in regulation of muscle contraction, the functional significance of the multiple TM isoforms present in nonmuscle cells has remained largely unclear. Several reports indicate that nonmuscle TMs serve important functions in microfilament stabilization [6, 7], regulating microfilament branching [8], actin polymerization [9], modulation of myosin functions [10], and intracellular transport [11]. These reports strongly suggest isoform-specific functions for nonmuscle TMs. Our work on the role of cytoskeletal proteins in cell transformation has demonstrated that derangements in TM expression are a common biochemical change in many breast carcinoma cells [12]. This observation extended the earlier findings that loss of TM expression is commonly found in many experimentally transformed fibroblasts [13–18]. Furthermore, it was demonstrated, using oncogene-transformed murine fibroblasts that restoration of tropomyosin isoform 1 (TM1) expression

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is adequate to revert the malignant phenotype induced by functionally diverse oncogenes, such as *ras* and *src* [19–21].

TMs are a family of closely related actin-binding proteins [17, 22]. Multiple isoforms of TMs are expressed from four genes via alternate splicing in a highly tissue-specific manner. The 284 amino-acid high M_r TMs generally display higher affinity to actin than the low M_r isoforms (248 aa) [22]. Other important cytoskeletal proteins, such as caldesmon, fascin, and tropomodulin, also modulate TM interactions with actin [23, 24].

Although it is known that suppression of high M_r TMs is a prominent feature of many experimentally transformed murine cell lines, the relevance of TMs in human cancers is largely unknown. To that end, we have investigated the role of TMs in mammary carcinogenesis. In normal human mammary epithelial cells, seven different isoforms of TMs are expressed [12]. Among these, TM1, TM2, TM3, and an epithelial cell type-specific species, TM38, may be categorized as high M_r TMs. Isoforms TM32a, TM32b, and another epithelial-specific protein, TM32, are known to be low M_r TMs. In spontaneously transformed human breast carcinoma cell lines, loss of expression of multiple isoforms of TMs has been reported. More significantly, expression of TM1 is completely abolished in all the transformed cell lines, suggesting that suppression of TM1 could be a pivotal event leading to the acquisition of the neoplastic phenotype by mammary epithelial cells.

The experimentally transformed fibroblasts employed in the above studies to define a causal relationship of TMs to cell transformation are generated by a single well-defined transforming oncogene. Most human cancers, on the other hand, originate in epithelial cells as a result of multiple genetic defects. Furthermore, mechanisms that lead to transformation of epithelial cells could be different and more complex. For example, while both raf and ras transform fibroblasts, epithelial cells can only be transformed by ras [25]. Another complexity with epithelial cells is that at least two more TM isoforms are expressed in epithelial cells compared to fibroblasts, which could potentially compensate for loss of the other TMs. Therefore, it remains to be established whether TM1 can function as a suppressor of the malignant phenotype of spontaneously transformed, human-derived carcinoma cells. To further investigate the role of TM1 in cellular transformation, we determined whether restoration of TM1 expression in MCF-7 human breast cancer cells has an effect on the growth and transformation status of these cells.

MATERIALS AND METHODS

Normal mammary epithelial MCF10A cells were obtained from Dr. Jose Russo, Fox Chase Cancer Center, Philadelphia [26], and MCF-7 and MDA MB 231 cells were purchased from ATCC. The cDNA encoding TM1 protein has been previously described [27]. Anti-TM polyclonal antiserum which recognizes multiple TMs, including TM1, was described previously [12]. DT/TM1 and DT/TM1-TM2 cells were fibroblast cell lines and previously described [28, 29]. MCF10A cells are used in this study as a reference for TM expression. Since MCF10A and MCF-7 (or those derived from MCF-7 cells) are not isogenic, no direct comparisons on the growth properties of these cells are made.

For retroviral gene transfer of TM1 into MCF-7 and MDA MB 231 cells, a pBNC recombinant virus was used as described [19, 30], except that an amphotrophic packaging cell line PA317 was used to generate the infectious virus. TMe1 cDNA was subcloned into a pBNC retroviral vector in which a CMV intron/enhancer drives the expression of the gene of interest and the selection is accomplished with G418. Transduced MCF-7 cells were cloned by a limiting dilution method and the cell lines (MCF-7/T) were tested for TM1 expression. For control purposes, MCF-7 cells were transduced with the empty vector and the resultant cell lines were designated MCF-7/V cells. Cell lines derived from transduction of MDA MB 231 are referred as MDA MB 231/T cells.

Protein analysis. Two-dimensional gel electrophoresis using cell lysates prepared from metabolically labeled cells was performed essentially as described previously [12, 30]. Western blotting was performed with TM antiserum or commercially available antibodies. Expression of α-tubulin was routinely measured with a specific antibody (Sigma Chemical Co.) as a load control. Antibodies against E-cadherin and catenins were purchased from Transduction Laboratories. For routine analysis, cells were extracted with a 50 mM Tris buffer, pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and a cocktail of protease inhibitors (1 mM PMSF and 1 μg/ml each of leupeptin, pepstatin, and aprotinin). After a brief centrifugation to remove nuclei and cell debris, the supernatant was used for immunoblotting or immunoprecipitations. These lysates were termed cytoplasmic preparations. Alternatively, cells were also extracted with RIPA buffer (RIPA lysates) [31]. To extract both nuclear and cytoplasmic proteins, cells were solubilized with 1% SDS and used for immunoblotting (whole cell lysates).

A 20-amino-acid sequence (187–206; SRARQLEEELRTMDQALKSL) that is distinctive for TM1 was used to generate a TM1-specific antibody in rabbits [27]. The antiserum was purified on a peptide immunoaffinity column and used for immunoblotting. It detects only TM1 on immunoblots. The polyclonal antiserum which recognizes all the TM isoforms has been previously described and was used for immunoblotting, immunocytochemistry and immunoprecipitations [28, 29].

Northern analysis. Total cellular RNA was analyzed as previously described [20]. The membranes were probed with a full-length TM1 cDNA [27]. The blots were washed at 65°C in a buffer containing 0.1× SSC and 1% SDS. Under these conditions, only TM1 is detected without any cross-hybridization to other TMs. Blots were then stripped and reprobed with β-actin for load controls.

Monolayer growth. Growth of the cells was measured in monolayer. Briefly, 2×10^5 cells were plated in normal (10%) serum containing media. At regular intervals, cells were harvested and counted using a hemocytometer. Cell culture conditions were previously described for MCF-7 and MDA MB 231 cells [26], and the medium for the transduced cells contained 200 μg/ml of G418. Experiments involving estrogen deprivation and supplementation were performed in a phenol red-free basal medium. Two replicate plates were initiated for each cell type and growth medium. To test the effects of estrogen on the growth of the cells, 72 h after plating, normal medium was replaced with a medium containing charcoal-stripped FBS alone (minus estrogen) or with a supplementation of 100 nM 17-βestradiol (plus estrogen). Cell count measurements were subsequently taken on each plate every 24 h (a total five measure-

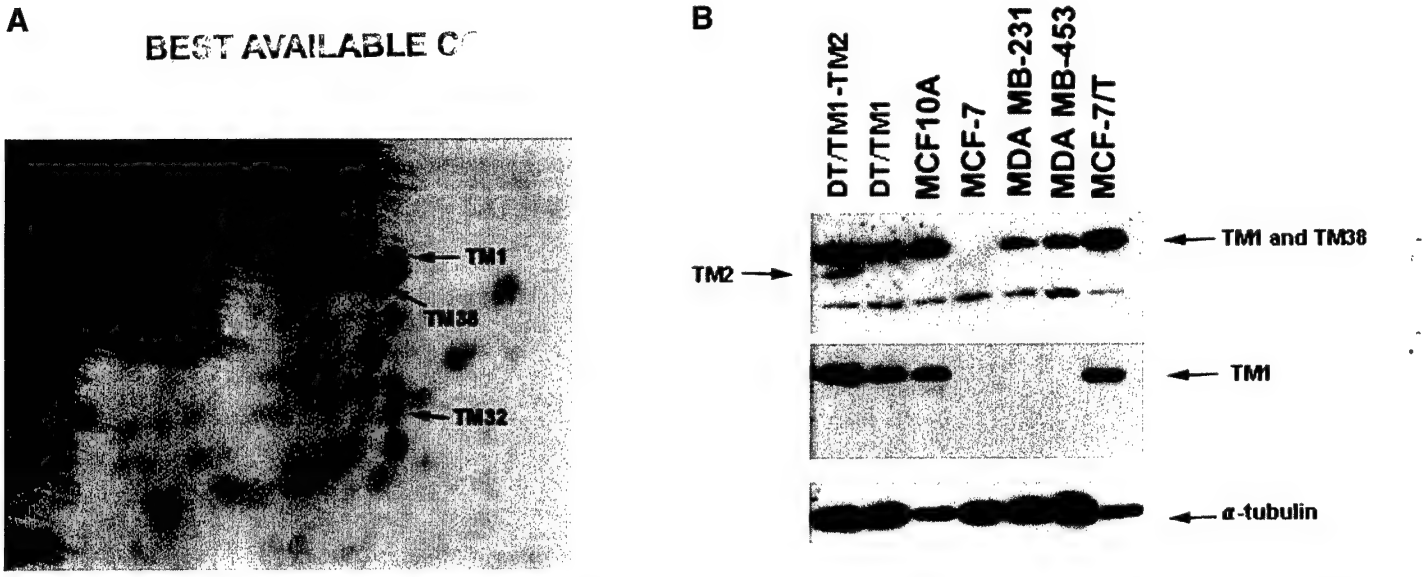

FIG. 1. TM expression in mammary epithelial cells. (A) Two-dimensional gel analysis of TM expression in MCF10A cells was performed as described under Materials and Methods. TM1, TM38, and TM32 are identified. (B) TM expression in normal and malignant breast cells. DT/TM1 and DT/TM1-TM2 cells were used as positive controls for TM expression. These are TM1-induced revertants of ras-transformed fibroblasts expressing either TM1 alone or both TM1 and TM2 [28]. TM2 migrates as a distinct band below TM1, which is evident in DT/TM1-TM2 cells (top). The cell lysates were probed with a polyclonal antiserum that reacts to multiple TMs (top), an antipeptide antibody that reacts specifically to TM1 (middle), or antitubulin antibody for load controls (bottom).

ments over 120 h). Mixed model analysis of variance (ANOVA) was performed, adjusting for estrogen exposure, to test for equality of growth after 120 h between the different cell types. Counts on the same plates were treated as a correlated measurements in the statistical analyses. Statistical analysis was performed using the SAS v8.1 software package (SAS, Cary, NC).

Anchorage-independent growth. Five or ten thousand cells were plated in soft agar in 35-mm petri dishes as described [19]. Cells were fed once in 48 h with 0.1 ml of medium. Six replicate plates were cultured for each cell type. Two weeks later, cells were stained with 0.05% nitroblue tetrazolium (Sigma). Colonies ≥ 50 μm were counted. Equality of the mean number of counted colonies after 2 weeks of incubation between the different cell types was tested using *t* tests.

Immunofluorescence. Cells were cultured in Nunc chamber slides, fixed with 3.7% paraformaldehyde [29], and permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS). For E-cadherin staining, samples were briefly extracted with 1% Triton X-100 and then fixed in paraformaldehyde, blocked, and reacted with primary antibody. The samples were blocked in 1% BSA (Jackson Immunolabs) in PBS and incubated with primary antibodies overnight at 4°C. The TM antiserum was used at 1:500, and other commercial antibodies were diluted according to the manufacturer's guidelines. The samples were also stained with Texas Red-conjugated phalloidin (Molecular Probes) for some experiments. The samples were mounted using the Prolong Antifade kit (Molecular Probes). Confocal microscopy was performed with a Zeiss confocal microscope with a 60X water objective. The images were optically sectioned and the composite images are projected.

For determination of the intensity of staining, the samples were viewed with a Zeiss Axioplan 2 microscope using either a 40X or 63X oil objective. The images were captured using a Dage MT1 camera (Model 300) and IFG 310 controller. The samples were photographed using different gate settings, which allows accumulation of different numbers of frames. The gate setting is inversely linked to the brightness of staining. For example, if a gate setting of 4 is used to photograph, four different individual frames will be taken and integrated in a final image. However, if a gate setting of 8 is required, it suggests that the image is about half as intensely stained as the first one. In these experiments, both the gain and black level, which affect the image quality, were kept at identical settings. The images were transferred to Adobe Photoshop and processed identically to make a composite image. Further quantification of the signal, such as area and intensity measurements using Photoshop, were not done. Multiple areas of the sample were photographed using gate settings of 4, 8, 16, and 32, depending on the intensity of the signal.

RESULTS

Restoration of TM1 expression in MCF-7 cells. Normal mammary and other epithelial cell types express seven different TMs, two more TMs than found in fibroblasts, that are readily detected in 2-D gels [12, 27]. TM expression in the nontransformed, immortalized human mammary epithelial MCF10A cell line is shown in Fig. 1A. One of these additional TMs, TM38, comigrates with TM1 in one-dimensional gels (Fig. 1B). The other TM isoform, TM32, is not resolved from the two low M_r TMs in routine SDS–PAGE, but requires 2-D analyses. Neither of these TM isoforms is well characterized. In malignant breast epithelial cells, TM1 expression was consistently absent, but the expression of other TMs including that of TM38 varies. For example, in MDA MB 231 and MDA MB 453 cells, TM38 expression is detectable by immunoblotting with polyclonal anti-TM antisera and by 2-D gels (Fig. 1B, top; [12]). However, in MCF-7 cells both TM1 and TM38 are lacking, and therefore no signal is detected.

The fibroblast cell lines DT/TM1 and DT/TM1-TM2, which express either TM1 alone or TM1 and TM2 together, respectively, are used as positive controls for TM1 expression. These cells lack the expression of TM38, and hence, the signal in the fibroblast-derived cell lines represents the expression of TM1 [12, 27, 28].

To facilitate the analysis of TM1 expression, we generated a specific antipeptide antiserum, as described under Materials and Methods. Data from immunoblotting with TM1-specific antibody are shown in Fig. 1B (middle). The two fibroblast cell lines DT/TM1 and DT/TM1-TM2 and the MCF10A cells contain readily detectable TM1. In agreement with two-dimensional gel analyses, expression of TM1 was lacking in MCF-7, MDA MB 231, and MDA MB 453 breast cancer cell lines [12]. In MDA MB 231 and MDA MB 453 cells which express TM38, no signal was detected by TM1-specific antibody, indicating the lack of TM1. MCF-7 cells that were transduced to reexpress TM1, designated MCF-7/T, however, were positive for TM1 expression (discussed below).

In MCF-10A human mammary epithelial cells, TM1 is expressed from a 1.1-kb mRNA. In MCF-7 human breast carcinoma cells, expression of both TM1 protein and its cognate mRNA is totally abolished, as is the case with several other breast carcinoma cells (Fig. 2A; also Fig. 1B) [12]. Transduction of MCF-7 cells with a recombinant retroviral vector results in the expression of a 2.0-kb mRNA from which TM1 is transcribed, as found in MCF-7/T cell lines; TM1 mRNA is 1.1 kb in size, while the additional sequences originate from the vector [19]. We have analyzed three independent MCF-7/T cell lines and two MCF-7/V cell lines along with the parental MCF-7 cells. In MCF-7/V cell lines (vector controls) and the parental MCF-7 cells, the transduced 2.0-kb mRNA is absent, as expected.

Immunoblotting of cytoplasmic lysates with a polyclonal anti-TM antibody revealed that TM1 is readily detectable in MCF10A cells. However, no corresponding signal was present in MCF-7 or MCF-7/V cells. In MCF-7/T cell lines, TM expression is restored (Fig. 1B, middle; data not shown for other cell lines). Since high M_r TMs do not resolve well in one-dimensional gels and the individual TMs significantly vary in their avidity to antibody, we examined total expression of TMs in these cell lines. To that end, cellular extracts prepared from metabolically labeled cells were analyzed by two-dimensional gels (Fig. 2B). In MCF-7 cells, expression of TM1, TM38, and TM2 is abolished, and therefore, none of these three proteins are detected [12]. Among the muscle-type high M_r TMs that are present in epithelial cells, only TM3 is present in MCF-7 and MCF-7/V cells. Transduction of MCF-7 cells with TM1 cDNA, as expected, results in a specific enhancement of TM1 protein in MCF-7/T cells. Furthermore, transduced TM1 is also found in the cytoskeletal fraction of MCF-7/T cells (data not shown).

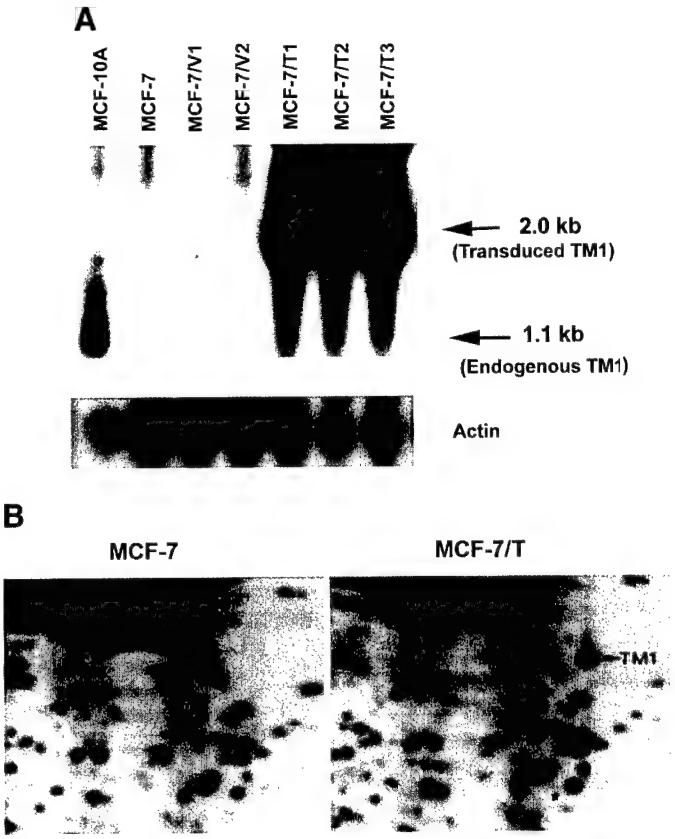

FIG. 2. Tropomyosin expression. (A) Northern blotting: Total cellular RNAs were probed with a full-length cDNA encoding TM1 at high stringency. Transduced TM1 is expressed as a 2.0-kb mRNA, while the endogenous TM1 is synthesized as a 1.1-kb RNA. The parental MCF-7 cells lack the mRNA encoding TM1. β-Actin was used for load controls (bottom). (B) Two-dimensional gel analyses of TM expression. Total cell lysates were prepared from pulse-labeled cells and analyzed by two-dimensional gels. TM1 is identified in the right panel.

Morphology of MCF-7/T cells. Restoration of TM1 expression in MCF-7 cells resulted in significant morphological changes. MCF-7 and the vector control cells shows that they grow as rather loosely adhering clusters. MCF-7/T cells in general grow in tighter clusters and form distinctive tubular structures (Fig. 3A) [32].

Immunocytochemical staining with anti-TM antibody of parental MCF-7 cells showed weak staining with the TM antiserum, although MCF-7 cells express at least one high M_r TM isoform and low M_r TMs. TM staining in MCF-7 cells is faint and diffuse throughout the cell, with no detectable association with actin filaments (Fig. 3B; A–C). In MCF-7/T cells, TM staining was intense, and TM containing microfilaments were evident. In addition, TM staining colocalized with that of actin, indicating that transduced TM1 reorganizes microfilaments (Fig. 3B; D–F). Although TM staining

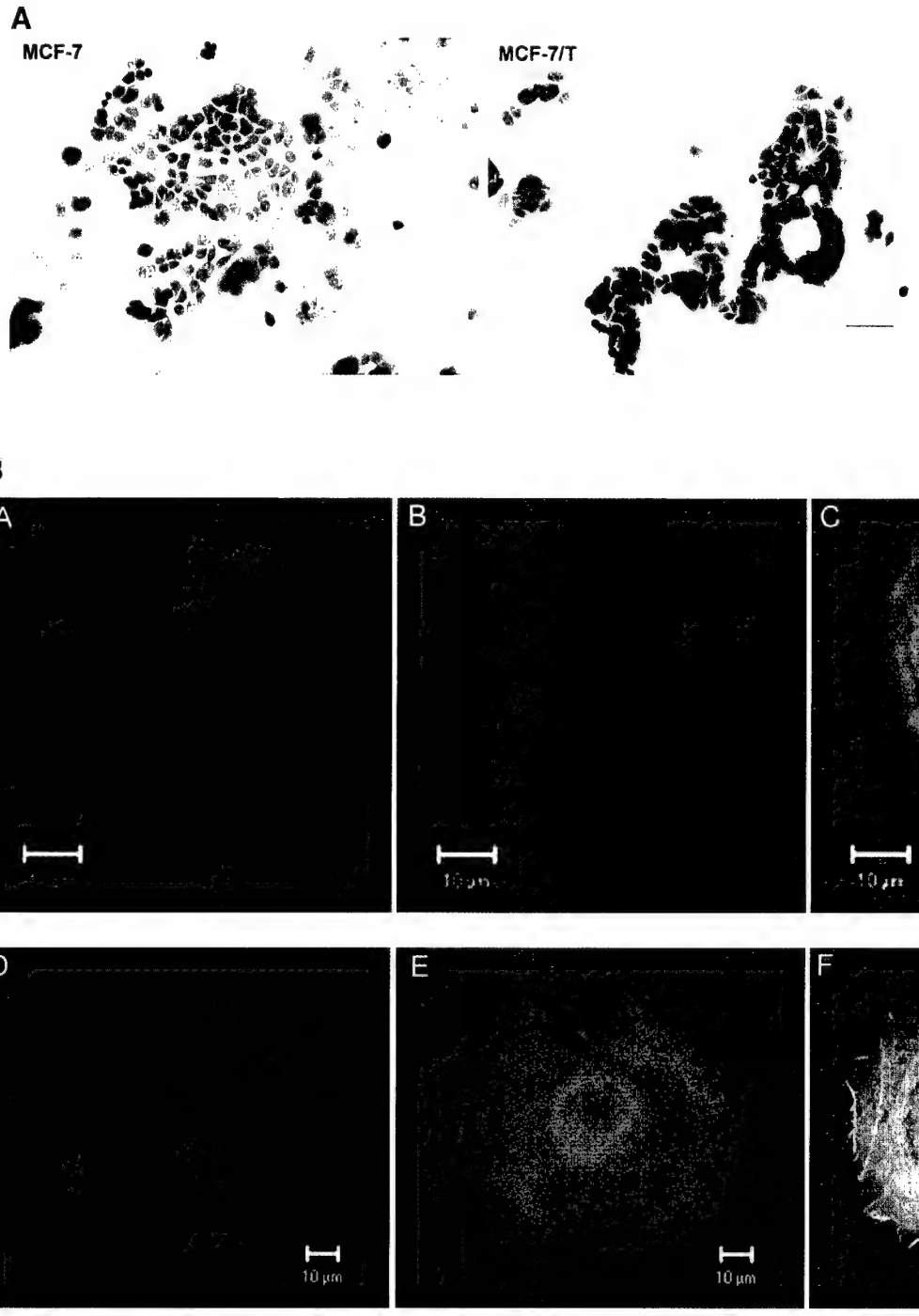

FIG. 3. (A) Morphology of TM1-expressing MCF-7 cells. Monolayers of MCF-7 and MCF-7/T cells were stained with H & E and photographed using an Olympus B20 microscope with a 2X objective. (B) TM1 associates with microfilaments. MCF-7 (A–C) and MCF-7/T (D–F) cells were immunostained with TM antiserum (A and D) followed by binding to FITC-conjugated antirabbit antibody (green) and Texas Red-conjugated phalloidin (B and E; red). Merged images (C and F) are presented. The samples were viewed using a confocal microscope.

was found through the cell body, it was brightest around the nucleus.

Growth properties. The effect of TM1 expression on the growth properties of MCF-7 cells was assessed in monolayer cultures. MCF-7, a vector control cell line (V1), and three cell lines expressing TM1 (T1, T2, and T3) were used to measure the growth. Under normal serum conditions, the unmodified MCF-7 and those transduced with empty vector grew rapidly at similar rates (Fig. 4A). However, all the three individual cell

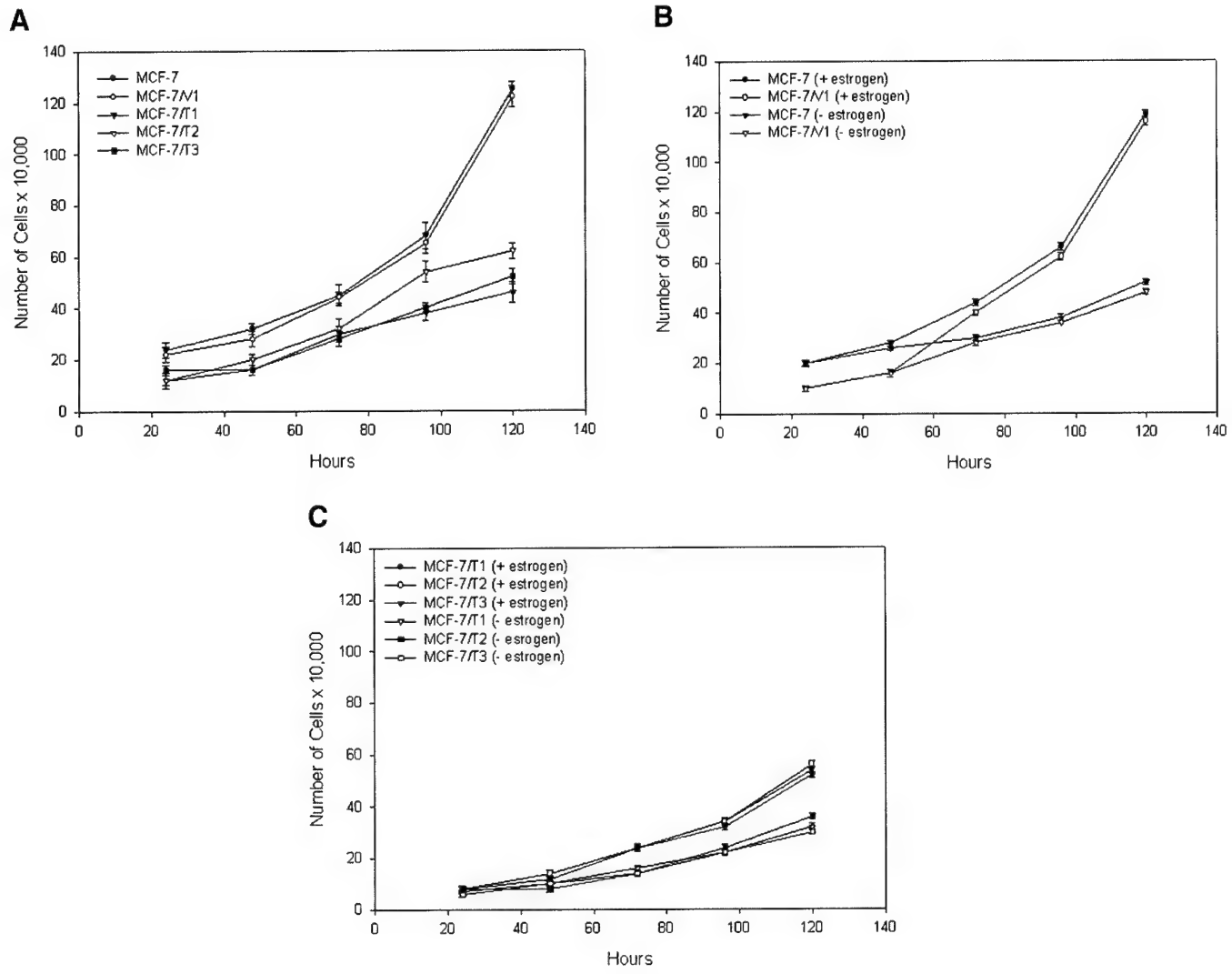

FIG. 4. TM1 expression decreases growth of MCF-7 cells. Cells were cultured under normal growth medium (A), in charcoal-stripped medium (estrogen deprivation), or in charcoal-stripped serum supplemented with 100 nM estradiol. The growth of MCF-7 and MCF-7/V (B), the three MCF-7/T cell lines (C), and under conditions of estrogen deprivation and supplementation is depicted. Error bars indicate standard deviation. The revertant cells grew significantly more slowly than the parental MCF-7 or vector control cells in normal serum, in the absence of or under estrogen supplementation.

lines expressing TM1 grew comparably, but demonstrated strikingly slower growth after 120 h than the parental MCF-7 or MCF-7/V cell lines ($P < 0.0001$ for all pairwise comparisons). Thus, restoration of TM1 expression decreases the proliferation of breast carcinoma cells.

Estrogen regulates the growth and differentiation status of MCF-7 cells. Since TM1 expression decreases the growth of these cells, we tested whether MCF-7/T cells remain sensitive to growth control by estrogen (Figs. 4B and 4C). When cells were cultured in the absence of estrogen using charcoal-stripped fetal bovine serum, growth of all the cell lines, including those expressing TM1, was inhibited by about 50%. Even under these conditions, MCF-7 and MCF-7/V1 cells (Fig. 4B) maintained higher growth than MCF-7/T cells, as shown in Fig. 4C ($P < 0.0001$ for all pairwise comparisons). Supplementation with 100 nM β-estradiol in charcoal-treated serum containing medium resulted in increased growth. In the presence of estrogen, MCF-7 and MCF-7/V1 cells demonstrated profoundly enhanced growth compared to MCF-7/T cells ($P < 0.0001$ for all pairwise comparisons). Addition of 4-hydroxytamoxifen inhibited the stimulatory effect of estrogen (data not shown). These data show that restoration of TM1 expression decreases the growth of MCF-7 cells, without altering the sensitivity to estrogen.

Anchorage-independent growth. The proliferation of normal cells is tightly regulated by growth signals of

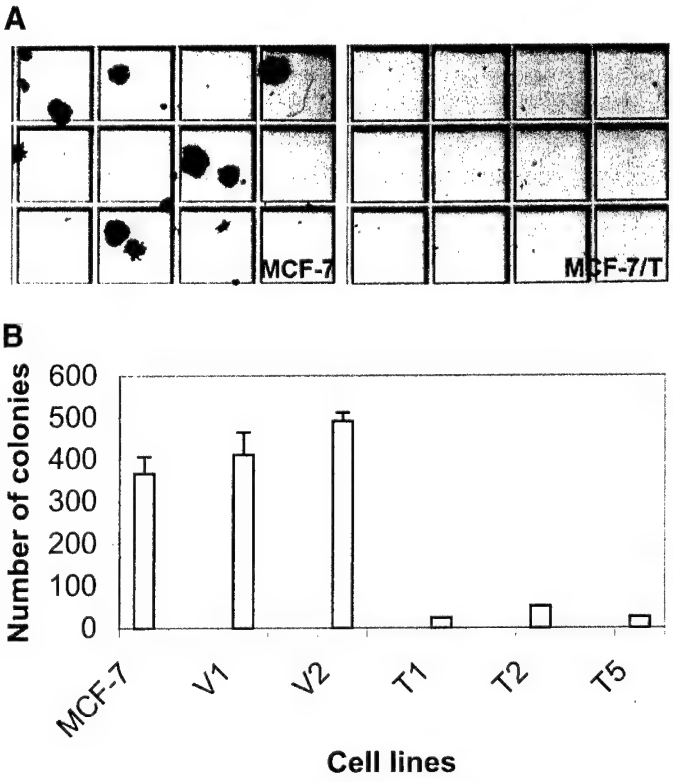

FIG. 5. TM1 suppresses anchorage-independent growth. Cells were plated in soft agar as described under Materials and Methods. At the end of the culture, they were stained with nitroblue tetrazolium, and photographed (A) and the number of colonies formed with each cell line is shown (B). Error bars indicate standard deviation.

integrin–extracellular matrix interactions, which is often deregulated in tumor cells. The ability of tumor cells to proliferate independent of adhesion closely correlates with tumorigenic potential, which is often assayed by anchorage-independent growth in soft agar. The data presented in Fig. 4 show that reexpression of TM1 decreases the growth of MCF-7 cells. Therefore, we examined whether TM1 inhibits anchorage-independent growth of MCF-7 cells. MCF-7 and V1 and V2 cells grew rapidly and formed a large number of colonies within 2 weeks (Figs. 5A and 5B). In contrast, the three clones of MCF-7 cells expressing TM1 did not grow in parallel cultures. The MCF-7/T cell lines showed a significant decrease in growth in the number of colonies ($P < 0.0001$ for all comparisons between MCF-7, V1, and V2 cells and MCF-7/T cells). These data demonstrate that reexpression of TM1 abolishes the anchorage-independent growth of breast carcinoma cells and support our earlier studies on the antitransformation effects of TM1.

Cell–cell adhesion molecules in TM1-transduced breast cancer cells. Expression of E-cadherin is downregulated in several human malignancies, including breast cancer. Decrease in E-cadherin expression weakens cell–cell interactions and contributes to the metastatic phenotype. Additionally, E-cadherin complexes with α, β, and γ catenins, and the entire complex is anchored to microfilaments. The cadherin–catenin complex is implicated in regulating tissue integrity, polarity, and differentiation. Stable association of the cadherin–catenin complex to microfilaments is considered a requirement for normal functioning of cadherin complexes [33, 34]. Furthermore, free, soluble β-catenin that is not associated with cadherins, is a key player in the wnt signaling pathways [35, 36]. Activation of wnt signaling pathways results in the transportation of β-catenin into the nucleus and interaction with TCF/LEF transcription factor, thereby upregulating gene expression. Since cells expressing TM1 form tighter clusters and display a tubular morphology indicating enhanced differentiation, we investigated whether TM1-induced reversion of breast carcinoma cells involves changes in the expression of E-cadherin or the catenins.

Cytoplasmic lysates were prepared from actively growing cells and were immunoblotted with antibodies against E-cadherin and α, β, and γ catenins. The data of Fig. 6A indicate that MCF-7 and the two vector control cell lines express similar levels of all the four proteins tested. The three MCF-7/T cell lines, however, showed significantly and consistently lower levels of E-cadherin and the catenins (Fig. 6A). Since TM1 expression is associated with the reemergence of microfilaments, we investigated a possibility that the cadherin complex may be more tightly associated with the cytoskeleton in the revertants, thus, forming stronger cell–cell junctions which may make them less soluble. To examine the total expression of these proteins, cells were extracted under more vigorous conditions, using RIPA buffer to lyse the cells, and tested for the presence of the components of the cadherin–catenin complex. Under these conditions, no detectable differences in the expression of E-cadherin or the catenins were found between the transformed (MCF-7 and V) and the revertant TM1-expressing MCF-7/T clones (Fig. 6B): similar results were obtained when cells were solubilized with 1% SDS and lysates were analyzed (data not shown). These results indicate that in MCF-7/T cell lines, E-cadherin and the catenins associate more tightly to the cytoskeleton, presumably contributing to the stability of cell–cell adherens junctions.

To investigate whether the localization of E-cadherin and the catenins is altered in the TM1-expressing cells, immunocytochemistry was performed. In MCF-7 cells, β-catenin (Fig. 7A; A) and E-cadherin (Fig. 7A; B) were detectable at the cell–cell junctions. In MCF-7 cells, E-cadherin's presence was evident in the perinuclear area as well as in the cytoplasm. In the revertant MCF-7/T cells, E-cadherin and β-catenin were also readily detectable at the cell adhesion areas with well-

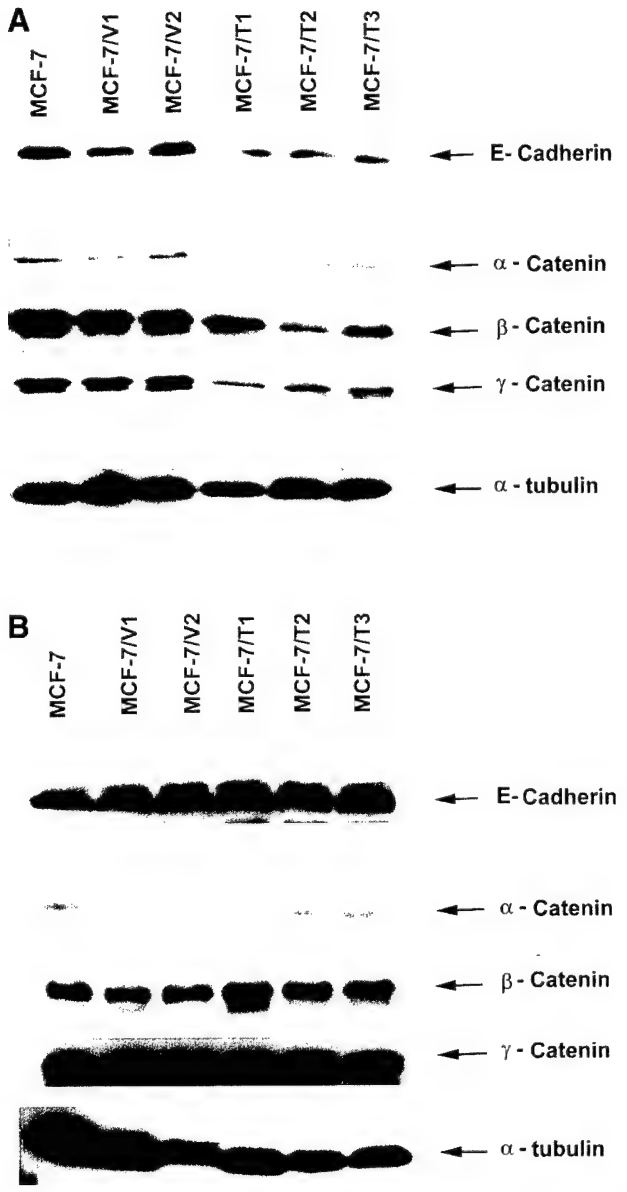

FIG. 6. The E-cadherin–catenin complex is more tightly associated in MCF-7/T revertant cells. Cytoplasmic lysates (A) and RIPA lysates (B) of the indicated cells were analyzed for expression of E-cadherin and the catenins by immunoblotting. Note that in the RIPA extracts, E-cadherin and the catenins are detected at comparable levels between the transformed and the revertant lines of MCF-7 cells.

defined staining (Fig. 7A; C and D, respectively). Further detailed analysis of the organization of these two molecules in the revertant cells revealed significant differences from the parental MCF-7 cells.

While both parental MCF-7 and MCF-7/T cells contained E-cadherin and β-catenin at cell–cell junctions, their association with the detergent-insoluble fraction was different between the cell types. The samples were stained with respective antibodies, visualized using a Zeiss fluorescence microscope, and photographed for different exposures as described under Materials and Methods. The samples were exposed to accumulate different number of frames, without any other adjustments to either black level or brightness settings. It was found that MCF-7 cells had significantly lower detergent-resistant E-cadherin and β-catenin at the cell–cell junctions (Fig. 7B, a and b) vis-à-vis MCF-7/T cells (Fig. 7B, c and d). When the samples were photographed for the same number of frames, the intensity of either E-cadherin and β-catenin at the cell–cell junctions was higher in MCF-7/T cells. MCF-7/T cells consistently retained higher amounts of E-cadherin and β-catenin at the cell–cell junctions than in MCF-7 cells. Multiple areas of both the cell types were examined at more than two gate settings. These data suggest that upon transduction of TM1, E-cadherin and β-catenin are more tightly linked to the cytoskeleton, which is in line with the immunoblotting results (Fig. 6). Under these conditions, α and γ catenins, however, were found to be at comparable levels in MCF-7 and MCF-7/T cells (data not shown).

Effects of restoration of TM1 on other breast cancer cells. TM1 expression is lacking in several other breast carcinoma cell lines [12]. To test whether TM1 functions as a suppressor in other breast carcinoma cell lines, or the anti-transformation effects of TM1 are limited to MCF-7 cells, we utilized the widely studied MDA MB 231 cells. MDA MB 231 cells are estrogen receptor-negative cells and highly invasive cells with fibroblastic features [37]. MDA MB 231 cells were transduced with TM1 and several cell lines (MDA MB 231/T cells) were isolated. Figure 8A shows immunoblotting with TM1-specific antibody and a polyclonal TM antibody of parental and transduced MDA MB 231 cell lysates. In agreement with the data of Fig. 1B, MDA MB 231 cells express only TM38, but not TM1, and accordingly a signal corresponding to TM38 was detected (Fig. 8A, middle). In two separate clones of MDA MB 231/T cells, transduced TM1 is detected with TM1-specific (Fig. 8A, top) and polyclonal antibody (Fig. 8A, middle). For comparison, we included MCF-7 and MCF-7/T1 cell lines, as TM1 is present in the latter cell line.

Next, we examined whether TM1 suppresses the anchorage-independent growth of MDA MB 231 cells. Two independent cell lines expressing TM1 and unmodified MDA MB231 cells were plated in soft agar and the colony formation was examined (Fig. 8B). The MDA MB 231 cells grew very rapidly and formed large colonies within 2 weeks, while the TM1-transduced cells did not show any growth even after 3 weeks in culture. These data indicate that restoration of TM1 expression is adequate to abolish a key attribute of transformed phenotype of MDA MB 231 cells. These

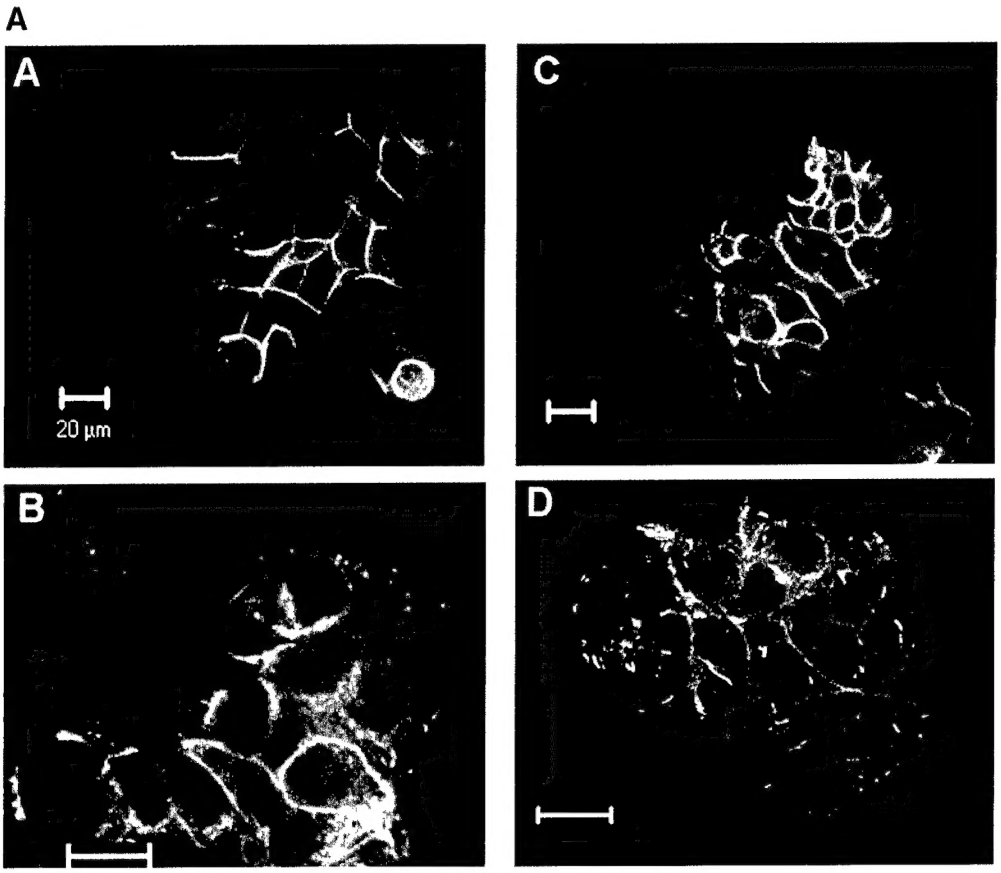

FIG. 7. (A) Organization of E-cadherin and β-catenin. MCF-7 (A and B) and MCF-7/T (C and D) cells were stained with either E-cadherin (A and C) or β-catenin (B and D) and examined by immunocytochemistry. In both cell types, the cell adhesion molecules are found at the cell–cell junctions. (B) E-cadherin and β-catenin are tightly associated at the cell–cell junctions of MCF-7/T cells. MCF-7 (a and b) and MCF-7/T (c and d) cells were stained with either E-cadherin (a and c) or β-catenin (b and d). The samples were exposed at different gate settings to accumulate different numbers of frames, which is dependent on the intensity of the signal. E-cadherin-stained samples were photographed at 16 frames, and β-catenin at 8 frames. Images at other higher or lower settings are not shown.

findings further support the hypothesis that TM1 is a suppressor of neoplastic transformation.

DISCUSSION

It is now widely recognized that normal functioning of the cytoskeleton is essential for maintaining normal growth and differentiation. For example, actin microfilaments are important determinants of cell morphology, cell motility, cell polarity, and cell division. In addition, reorganization of actin microfilaments occurs in response to activation of integrins and it is likely to play an important role in "inside out" signaling [5]. The attachment of cadherin–catenin complexes to microfilaments is known to strengthen cell–cell adhesion and contribute to tissue integrity. Furthermore, more recent data suggest that many signaling molecules are linked to microfilaments [38, 39]. Transformed cells generally lack well-defined microfilament bundles which, besides being a cause for loss of normal morphology, are potentially important contributing factors for metastatic behavior [3].

Several lines of evidence presented herein support the thesis that TM1 is a suppressor of the transformed phenotype of breast cancer cells: (i) restoration of TM1 expression results in the growth of MCF-7 cells as tighter colonies with a more glandular morphology, while the parental and wild-type MCF-7 cells grow as more scattered colonies; (ii) TM1 expression leads to significantly decreased growth in monolayer cultures; and (iii) TM1 expression completely abolishes the anchorage-independent growth of two spontaneously transformed breast cancer cells, viz. MCF7 and MDA MB 231 cells. Earlier work from this laboratory has demonstrated that TM1 reverts the transformed phenotype of *Ki-ras-* or *v-src*-transformed fibroblasts. Taken together, these findings support the notion that TM1 could be a general suppressor of malignant transformation.

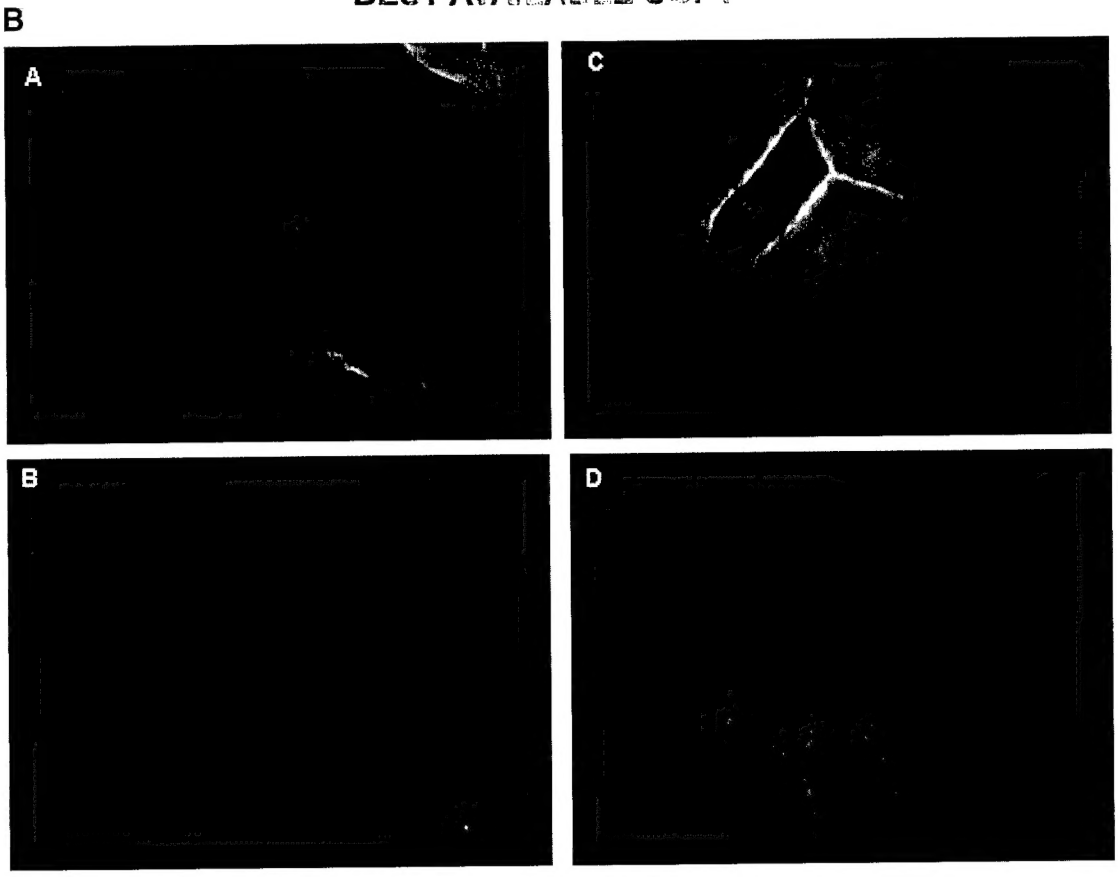

FIG. 7—*Continued*

Many key questions, however, remain regarding the role of TM1 in malignant transformation. First, although TM1 expression has been shown to be downregulated or lacking in many tumor cell lines, it is not clear whether the expression of TM1 is lost in primary tumor tissues. A main reason for this gap in understanding the role of TM1 in cellular transformation is the lack of suitable reagents to assess TM1 expression specifically in tissues where multiple TM isoforms are expressed. The downregulation of TM1 expression in breast and other cancer cells appears to be mediated by TM1 promoter methylation and histone deacetylation [40].

Second, the mechanism of TM1-mediated suppression of transformed growth is poorly understood. TM1 is a structural protein and does not possess the functional domains that may mediate specific protein–protein interactions, which could readily explain its role as a suppressor of transformation. TM1, like other TMs, is an actin-binding protein, and we find that TM1 alone restores microfilament architecture and suppresses the transformed growth phenotype: other TMs, such as TM2, cooperate with TM1 in reorganizing the cytoskeleton [28]. We investigated the possibility that the TM1-induced cytoskeleton could be important in the reversion of transformed growth.

Cadherin–catenin complexes mediate cell–cell adhesion and a number of studies have demonstrated that the integrity and functioning of these cell adhesion complexes are disrupted in many types of cancers, including those originating in breast [41–43]. E-cadherin, often referred to as a metastasis suppressor, is either generally expressed at low levels [2, 44] or mutated in some breast cancer specimens [45, 46]. The interactions of cadherin–catenin complexes with the cytoskeleton are important in maintaining normal adhesion via cadherins [33, 34].

In TM1-expressing revertants of MCF-7 cells, while there appear to be no qualitative differences, E-cadherin and the catenins are more tightly coupled to cytoskeleton, as evident from the differential extractability of these proteins. Enhanced detergent solubility of E-cadherin and β-catenin is more frequently observed in transformed cells, which may result in the assembly of defective adhesion junctions. In TM1-expressing revertant cells, E-cadherin and β-catenin are more tightly associated with the cytoskeleton, as evidenced by immunoblotting studies (Figs. 6 and 7) and immunofluorescence experiments. Consistent with these data, it was reported that ras transformation of breast epithelial cells does not change the expression of

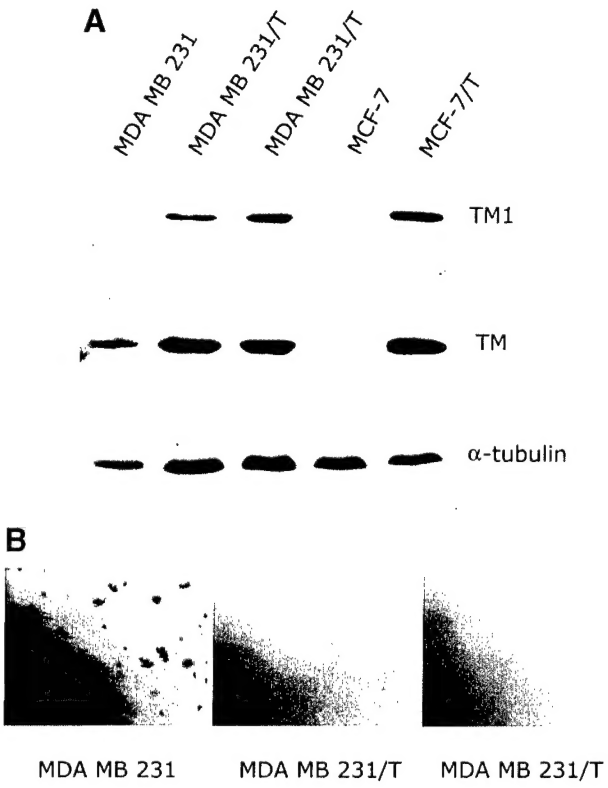

FIG. 8. TM1 expression suppresses the anchorage-independent growth of MDA MB 231 cells. (A) TM1 expression in MDA MB 231 cells and TM1-transduced cells was determined by immunoblotting with TM1-specific (top) and TM polyclonal (middle) antibodies, as described in Fig. 1B. (B) Ten thousand cells of MDA MB 231 and two MDA MB 231/T cells were cultured in soft agar for 3 weeks, and the colonies were enumerated as described under Materials and Methods. While MDA MB 231 cells grew in soft agar and formed 3660 colonies, with MDA MB 231/T cells no growth was detected.

E-cadherin or β-catenin, but results in the increased detergent solubility of these molecules [31]. Increased association with the cytoskeletal elements is also reflected in the localization of E-cadherin and β-catenin to cell–cell boundaries of the revertant cells. Current efforts are directed at investigating the biochemical basis of these interactions.

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